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<b>(21) International Application Number:</b> PCT/NZ97/00107 <b>(22) International Filing Date:</b> 29 August 1997 (29.08.97)  <b>(30) Priority Data:</b> 08/713,557      30 August 1996 (30.08.96)      US  <b>(71) Applicant:</b> GENESIS RESEARCH & DEVELOPMENT CORPORATION LIMITED [NZ/NZ]; 1 Fox Street, Parnell, Auckland (NZ).  <b>(72) Inventors:</b> WATSON, James, D.; 769 Riddell Road, Auckland (NZ). RUDERT, Fritz; 1/53 Amy Street, Ellerslie, Auckland (NZ).  <b>(74) Agents:</b> BENNETT, Michael, Roy et al.; Russell McVeagh West-Walker, Level 5, The Todd Building, 171-177 Lambton Quay, Wellington 6001 (NZ).		<b>(81) Designated States:</b> AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).  <b>Published</b> <i>Without international search report and to be republished upon receipt of that report.</i>
<b>(54) Title:</b> CD95 REGULATORY GENE SEQUENCES AND TRANSCRIPTION FACTORS  <b>(57) Abstract</b> <p>Regulatory DNA sequences that silence and enhance transcription of coding portions of the CD95 gene, which is instrumental in apoptosis, are disclosed. Proteinaceous transcription factors that bind to the silencer and enhancer regulatory sequences are also disclosed and are useful for modulating the expression of CD95 or other proteins. Methods for regulating apoptosis have therapeutic and prophylactic applications for a variety of disorders, including cancer, viral and retroviral infections, neurodegenerative disorders, immune system dysfunction, and other disorders.</p>		

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## CD95 REGULATORY GENE SEQUENCES AND TRANSCRIPTION FACTORS

Field of the Invention

5 The present invention relates, generally, to regulation of expression of a gene encoding the CD95 receptor, which plays an important role in apoptosis, or programmed cell death. More specifically, the present invention relates to regulation of CD95 gene expression through identification of regulatory sites on the CD95 gene, proteinaceous transcription factors that bind to the CD95 regulatory sites, and methods for regulating CD95 gene transcription and expression.

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Background of the Invention

Apoptosis is a cell suicide mechanism that is used by multicellular organisms to regulate physiological cell death for purposes of defense, development, homeostasis and aging. Apoptosis is an active process modulated by its own  
15 regulatory system and genetics and is generally characterized by morphological changes including loss of contact of a cell with its neighbors, chromatin condensation, membrane blebbing, cytoplasmic condensation, DNA fragmentation and, eventually, the generation of membrane-enclosed apoptotic bodies that are phagocytosed by neighboring cells.

20 CD95 (also referred to as Fas or APO-1) is an important receptor in apoptosis. Apoptotic cell death is triggered by an interaction of the CD95 receptor with its ligand CD95L. CD95 is a member of the tumor necrosis factor (TNF) receptor family of cell surface proteins, and CD95L is a member of the TNF family of membrane and secreted proteins. CD95 is expressed on a wide variety of cell  
25 types, either constitutively or inducibly. CD95 is expressed, for example, on activated T and B cells, and its mRNA has been detected in other tissues including thymus, spleen, liver, ovary, lung, and heart.

CD95 has been implicated in mediating nonspecific T-cell cytotoxicity and activation-induced cell death (AICD) in the peripheral immune system. When  
30 apoptosis is induced in T cells by activation through an antigen receptor, signals are passed into the cell, leading to activation of the cell and expression of *c-myc*. The cell up-regulates both CD95 and CD95L and expresses them on the cell surface.

These molecules then interact with each other, in an autocrine or paracrine manner, initiating the cell death-inducing signaling pathway. Overexpression of CD95 receptor signaling domain results in apoptosis and cell death.

Regulating apoptosis has therapeutic and/or prophylactic implications for diseases where apoptosis causes the pathology, including chronic neurodegenerative disorders such as Alzheimer's and Parkinson's diseases and multiple sclerosis, and immunosuppressive disorders, both genetic and acquired. Similarly, regulating apoptosis may have therapeutic benefits under circumstances in which apoptosis occurs as a result of trauma, such as strokes and heart attacks. Agents that block apoptosis may be useful in treating ischemic conditions, such as heart attacks, strokes or reperfusion injury, by blocking the apoptotic response in cells. Pathological suppression of apoptosis appears to be an important factor in neoplastic diseases and viral infection. Apoptosis is suppressed, for example, in proliferating tumor cells. HIV/AIDS infection produces unregulated and untimely apoptosis in crucial defenders of the immune system, namely CD-4 cells. Moreover, modulation of apoptosis may increase tolerance to pharmaceutical agents such as chemotherapeutic and radiotherapeutic agents that stress but, in the absence of an apoptotic mechanism, may not kill cells. Regulation of apoptosis may also have implications for *in vitro* cell growth and maintenance and may be used to produce more robust cell lines and increase production of recombinant proteins.

The numerous applications in which regulation of apoptosis may play an important role underscore the importance of developing a more complete understanding of expression of the CD95 receptor. Identification of regulatory sequences on the CD95 gene, as well as transcription factors that bind to such regulatory sequences, will provide means for modulating transcription and expression of this important receptor, thereby providing a means to regulate apoptosis.

#### Disclosure of the Invention

In one aspect, the present invention provides novel, isolated and purified polynucleotides that are involved in transcriptional regulation of the CD95 receptor and variants thereof which possess similar regulatory properties. Polynucleotides

that play a role in enhancing and silencing transcription from the CD95 promoter are disclosed. Regulatory polynucleotides of the present invention are located in a 70bp region about 1 kb upstream from the coding portion of the CD95 gene. Presently preferred polynucleotides that function as regulatory elements in the enhancement of transcription from the CD95 promoter are described in SEQ ID NOS: 1 and 37. The polynucleotide identified as SEQ ID NO: 1 exhibits enhancing regulatory activity, but contains both enhancer and silencer regulatory elements. The polynucleotide identified as SEQ ID NO: 37, which exhibits enhancing but not silencing regulatory activity, is especially preferred as an enhancing regulatory element. A presently preferred polynucleotide that functions as a regulatory element in silencing transcription from the CD95 promoter is described in SEQ ID NO: 2.

In another aspect, the present invention discloses novel isolated and purified polynucleotides that provide sites for binding of transcription factors that regulate transcription from the CD95 promoter. All or a portion of the polynucleotide sequences described in SEQ ID NOS: 1 and 2 provide binding sites for transcription factors that modulate transcription of coding portions of the CD95 gene. A presently preferred polynucleotide sequence consensus motif that provides a binding site for transcription factor(s) that enhance transcription from the CD95 promoter is set forth in SEQ ID NO: 3. Additional preferred polynucleotides that provide sites for binding of transcription factors that enhance transcription from the CD95 promoter are set forth in SEQ ID NOS: 4, 5, 6 and 37. Additional preferred polynucleotides that provide sites for binding of a transcription factor that silences transcription from the CD95 promoter are set forth in SEQ ID NOS: 7 and 36.

Yet another aspect of the present invention relates to proteinaceous binding molecules, or polypeptides, referred to as transcription factors, that bind specifically to the CD95 silencer and enhancer regions described above to enhance or inhibit transcription of the CD95 gene. Binding of an enhancer transcription factor to an enhancer regulatory region stimulates CD95 expression; binding of a silencer transcription factor to a silencer regulatory region inhibits CD95 expression. The term "CD95 transcription factor," as used herein, refers to any one of a series of polypeptides which are capable of binding to polynucleotides that regulate transcription and/or expression of CD95. CD95 transcription factors derived from

human, as well as other mammalian species, and partially or wholly synthesized polypeptides are within the scope of this invention.

Transcription factors that bind to polynucleotide probes corresponding to the enhancer regulatory sequences described above (SEQ ID NOS: 1, 3-6 and 37) form distinct DNA/polypeptide complexes having molecular weights of approximately 59 kDa, 113 kDa and 200-300 kDa. Experimental evidence demonstrates that transcription factors capable of binding to CD95 enhancer regulatory polynucleotides exhibit double stranded binding activity. Transcription factors that bind to polynucleotide probes corresponding to all or a portion of the silencer regulatory polynucleotide sequences (SEQ ID NOS: 2, 7 and 36) form distinct DNA/polypeptide complexes having molecular weights of approximately 47 kDa, 77 kDa and 100 kDa. Experimental evidence demonstrates that transcription factors capable of binding to CD95 silencer regulatory polynucleotides exhibit single stranded binding activity.

Several specific transcription factors that bind to CD95 regulatory polynucleotides and thereby modulate expression of CD95 have been identified experimentally. Polypeptide transcription factors that bind to the CD95 silencer regulatory polynucleotide sequences include human YB-1 (EMBL Accession No. M24070, SWISS-PROT Accession No. P16990); rat YB-1 (EMBL Accession No. M57299, SWISS-PROT Accession No. P22568); rat Pura (SEQ ID NOS: 38, 39, 41 and 42); and a rat Pura-like protein (SEQ ID NOS: 40 and 43). Binding of these silencer transcription factors to a CD95 silencer regulatory polynucleotide inhibits expression of CD95 and thereby tends to inhibit apoptotic cell death. Polypeptide transcription factors that bind to the CD95 enhancer regulatory polynucleotide SEQ ID NO: 11 include human YB-1 and human hnRNP D. Binding of this enhancer transcription factor to a CD95 enhancer regulatory polynucleotide stimulates expression of CD95 and thereby tends to stimulate apoptotic cell death.

YB-1 is the 47 kD protein species observed by Southwestern and UV-crosslinking analysis with the CD95 silencer probe (SEQ ID NO: 2). YB-1 is also known as human dbpB or CCAAT-binding transcription factor 1 subunit A (CBF-A) or EF1a or MDR NF-1 (EMBL Accession No. M24070; SWISS-PROT Accession No. P16990); bovine EF1A#1 (EMBL Accession No. M95793; SWISS-

PROT Accession No. P16990); rat dbpB or EF1a or CBF-A (EMBL Accession No. M57299; SWISS-PROT Accession No. P22568); murine CBF-A or MUSY1 or MSY1 or MUSYB (EMBL Accession No. M60419; SWISS-PROT Accession No. P27817) or MYB-1A (EMBL Accession No. U33196; SWISS-PROT Accession No. Q60950) or MYB-1B (EMBL Accession No. U33197; SWISS-PROT Accession No. Q60951); rabbit MRNP p50 (EMBL Accession No. U16821; SWISS-PROT Accession No. Q28618); avian EF1a or RSV-EF-1 (EMBL Accession No. L13032; SWISS-PROT Accession No. Q06066) or pYB $\alpha$  (EMBL Accession No. U43513; SWISS-PROT Accession No. Q90376); and frog FRGY1 (EMBL Accession No. M59453; SWISS-PROT Accession No. P21573), and has been described as a 42-50 kD protein. YB-1 is a member of a highly conserved nucleic acid-binding polypeptide family containing a cold-shock domain known as the Y-box family of proteins. The cold shock domain of the Y-box nucleic acid-binding polypeptide family has been identified as a 66 amino acid region, is believed to be the DNA-binding domain, and is highly conserved. These proteins are described in Wolffe et al. (1992), The Y-box factors: a family of nucleic acid binding polypeptides conserved from *Eschericia coli* to man, *New Biol.* 4, 280-298.

Other members of the Y-box family of binding proteins that contain a cold-shock domain include human dbpA (EMBL Accession No. M24069; SWISS-PROT Accession No. P16989); human dbpA-like protein (EMBL Accession No. X95325; SWISS-PROT Accession No. Q14121), human dbpB-like protein (EMBL Accession No. L28809; SWISS-PROT Accession No. P16990), human NSEP-1 (EMBL Accession No. M83234; SWISS-PROT Accession No. Q14972), and human nuclease sensitive element binding protein-1 (EMBL Accession No. M85234; SWISS-PROT Accession No. Q15325); rat YB-2 (EMBL Accession No. U22893; SWISS-PROT Accession No. Q62764) and rat RYB-a; murine dbpA (EMBL Accession No. D14485; SWISS-PROT Accession No. Q61478); frog FRGY2 or p56 or MRNP4 or YB-2 (EMBL Accession No. M59454; SWISS-PROT Accession No. P21574) and frog p54 or MRNP3 (EMBL Accession No. M80257, SWISS-PROT Accession No. P45441); and bacterial cspA and cspB. Many other members of the nucleic acid-binding Y-box polypeptide family having a cold shock domain, such as proteins from lower organisms, also exhibit homology and would bind to the

regulatory polynucleotides of the present invention. Polypeptides having a cold shock domain having at least 95% and preferably at least 98% identity to the cold shock domain of human YB-1 (EMBL Accession No. M24070; SWISS-PROT Accession No. P16990) are considered to be members of the Y-box family of binding polypeptides and are especially preferred for use in compositions and methods of the present invention.

YB-1 binds to both double-and single-stranded DNA, although it has been shown to preferentially bind single-stranded DNA. It has also been shown to induce or stabilize a single-stranded conformation in DNA. YB-1 has been described as a transcription factor for a number of genes and, in particular, as an activator of a number of growth-associated genes, including epidermal growth factor (EGF) receptor gene and proliferating cell nuclear antigen (PCNA)/cyclin gene. It has also been shown to be an activator of the human multidrug resistant pump (mdr1) gene. YB-1 has also been shown to be a repressor of the MHC Class II genes and to stimulate transcription of the LTRs of both the human T-cell lymphotropic virus-1 (HTVL-1) and human immunodeficiency virus-1 (HIV-1). A number of tumor cells have very high levels of CD95L mRNA, and negligible levels of CD95 mRNA (e.g. lung, colon, liver and skin carcinomas). These levels are the inverse of what is found in non-malignant cells. It is believed that the lack of CD95 expression allows tumors to evade the cytolytic T-cells through the expression of CD95L, so inducing apoptosis of the activated T-cells. Furthermore, the levels of mdr and/or PCNA and/or EGF receptor mRNAs are often higher in tumor cells than non-malignant cells. YB-1 activates expression of all of these genes and, at the same time, represses CD95 gene transcription. This suggests that YB-1 and the Y-box family of proteins are involved in the biology of malignancies. Modulation of CD95 gene transcription through modulation of the binding of a Y-box protein to the identified CD95 silencer polynucleotides, renders the activated T-cells less susceptible to apoptosis and, at the same time, confers resistance to cytotoxic drugs. Modulation of expression of CD95 may also be used to promote apoptosis of malignant cells by means of an auto-induced or T-cell mediated mechanism.

Pur $\alpha$ , also known as ssCRE-BP or SPSF I is a 34-42 kD protein that has single-stranded DNA-binding activity. Human Pur $\alpha$  (EMBL Accession No.



M96684; SWISS-PROT Accession No. Q00577) and murine Pura (EMBL Accession No. U02098; SWISS-PROT Accession No. P42669) have been fully sequenced. Human, murine and rat Pura are highly conserved; human and murine Pura are 99.3% identical. Pura often occurs as a dimer or oligomer.

5        The 5' portion of the rat Pura transcription factor isolated and identified by applicants is encoded by the polynucleotide identified as SEQ ID NO: 38, and the 3' portion of the rat Pura transcription factor isolated and identified by applicants is encoded by the polynucleotide identified as SEQ ID NO: 39. The polypeptides encoded by the polynucleotides set out in SEQ ID NOS: 38 and 39 are identified in  
10    SEQ ID NOS: 41 and 42, respectively. Polypeptides having at least 95% identity and preferably at least 98% identity to human Pura (EMBL Accession No. M96684; SWISS-PROT Accession No. Q00577) are preferred for use in the compositions and methods of the present invention. The polypeptides identified in SEQ ID NOS: 41 and 42 are also encompassed within the term "Pura," and transcription factors  
15    comprising the polypeptides identified in SEQ ID NOS: 41 and 42 are also preferred for use in compositions and methods of the present invention.

Pura has been shown to be a factor involved in cell cycle control of DNA replication and also transcription. Like YB-1 mRNA, Pura mRNA has been found in every tissue examined. As a transcription factor, Pura has been implicated in the  
20    control of transcription of genes activated by two different retroviruses – human polyoma virus (JCV) and the Rous sarcoma virus (*v-src*). Pura interacts with YB-1 to bind to sequences of the JCV enhancer and it is believed that this may induce the shift from early to late viral gene transcription. Pura has also been shown to interact with the human immunodeficiency virus-1 (HIV-1) Tat protein to activate  
25    transcription of JCV. Tat has been implicated in modulating CD95-mediated apoptosis of CD4<sup>+</sup> T cells in HIV-1-infected persons. Thus, an interaction of Tat and Pura may contribute at the transcriptional level to modulate the expression of CD95 in HIV-1 patients. Pura has also been shown to associate with the Retinoblastoma protein (Rb), which is encoded by a tumor suppressor gene.

30        Another CD95 silencer binding polypeptide having a molecular weight of 32kD, referred to as "Pura-like transcription factor" has been identified. The 5' portion of the polynucleotide encoding the Pura-like polypeptide is provided in SEQ

ID NO: 40. The N-terminal amino acid sequence of the Pura-like transcription factor encoded by the polynucleotide identified as SEQ ID NO: 40 is provided in SEQ ID NO: 43. It is believed that this is a novel polypeptide transcription factor. Transcription factors comprising the polypeptide identified in SEQ ID NO: 43 and  
5 proteins having at least 95% identity and preferably at least 98% identity to the polypeptide identified in SEQ ID NO: 43 are encompassed within the term "Pura-like transcription factor(s)" and are preferred for use in compositions and methods of the present invention.

Two polypeptide transcription factors were identified that bind to the CD95  
10 enhancer regulatory sequence and thereby modulate CD95 expression: human YB-1 and human hnRNP D (EMBL Accession No. D55672; SWISS-PROT Accession Nos. Q14101 and Q14103). Human YB-1 is described above. HnRNP D is also known as hnRNP D0 and AUF1 (EMBL Accession No. A54601; SWISS-PROT Accession No. Q12772), and is a heterogeneous nuclear ribonucleoprotein having a  
15 molecular weight of 33 kD. Polypeptides having at least 95% identity and preferably at least 98% identity to human hnRNP D (EMBL Accession No. D55672; SWISS-PROT Accession Nos. Q14101 and Q14103) are preferred for use in the compositions and methods of the present invention. HnRNP D has been described as a UUAG-specific RNA-binding protein. Another heterogeneous nuclear  
20 ribonucleoprotein, hnRNP K, has recently been shown to be a transcription factor which activates expression of the c-myc gene promoter.

Another aspect of the present invention relates to use of the regulatory polynucleotides and/or transcription factors of the present invention to modulate transcription of the CD95 gene, as well as genes other than CD95. Modulation of  
25 transcription of the CD95 gene may be achieved, for example, by blocking the binding of transcription factors to the enhancer or silencer regulatory regions, by modulating expression of one or more transcription factors, by modulating the binding activity of one or more transcription factors, or by modulating the functional activity of one or more of the transcription factors. Modulation of transcription of  
30 the CD95 gene may be implemented to treat various conditions and disease states by selectively stimulating or inhibiting apoptotic cell death.

Blocking of the binding of transcription factors to CD95 regulatory regions may be accomplished, for example, by introducing small molecules, such as oligodeoxyribonucleotides, synthetic polyamides, or other small molecules that target specific CD95 regulatory sequences to control gene expression by preventing the binding of transcription factors. Specifically, introduction of small molecules that target and bind to, or otherwise associate with, the regulatory sequences identified as SEQ ID NOS: 1-7, 36 and 39 to inhibit the binding of transcription factors are preferred. The identification and development of suitable small molecules is described, for example, in Cai et al. (1996), Transcription-modulating drugs: mechanism and selectivity, *Curr. Opin. Biotechnol.* 7 (6): 608-615 and Gottesfeld et al. (1997), Regulation of gene expression by small molecules, *Nature* 387 (6629) 202-205.

Blocking of the binding of transcription factors to CD95 regulatory regions may additionally or alternatively be accomplished, for example, using oligonucleotide-directed triple helix formation. The use of such methods is documented, for example, in Maher, L.J. (1992), DNA triple-helix formation: an approach to artificial gene repressors, *Bioessays* 14 (12): 807-815 and Chan et al. (1997), Triplex DNA: fundamentals, advances and potential applications for gene therapy, *J. Mol. Med.* 75 (4) 267-282.

Alternatively, transcription factors may be inhibited from binding to CD95 regulatory regions by introducing an excess of one or more polynucleotides, specifically polynucleotides comprising the regulatory sequences identified as SEQ ID NOS: 1-7, 36 and 37. Excess polynucleotides may be introduced using a variety of techniques which are well known in the art. Overexpressing a polynucleotide comprising a YB-1 transcription factor binding sequence (SEQ ID NOS: 2 or 36) in cells, for example, would result in binding of endogenous YB-1 to the introduced polynucleotides. There would consequently be insufficient endogenous YB-1 to bind to the CD95 silencer regulatory region, which would result in activation of the transcription of CD95.

Modulating the expression of transcription factors may be accomplished, for example, by inhibiting translation of the relevant transcription factors. Translation of the relevant transcription factors may be inhibited, for example, by introducing anti-

sense expression vectors; by introducing antisense oligodeoxyribonucleotides or antisense phosphorothioate oligodeoxyribonucleotides; by introducing antisense oligoribonucleotides or antisense phosphorothioate oligoribonucleotides; or by other means which are well known in the art. The use of techniques involving antisense polynucleotides is well known in the art and is described, for example, in Robinson-  
5 Benion et al. (1995), *Antisense techniques, Methods in Enzymol.* 254 (23): 363-375 and Kawasaki et al. (1996), *Artific. Organs* 20 (8): 836-848.

Desired transcription factors may alternatively be overexpressed by introducing DNA constructs that code for a desired enhancer or silencer transcription  
10 factor to increase the population of the desired transcription factor. Suitable DNA constructs comprise a polynucleotide encoding the relevant transcription factor and suitable promoter and terminator sequences. Specifically, preferred polynucleotides for use in such DNA constructs include polynucleotides encoding a member of the Y-box family of binding proteins, polynucleotides encoding a Pur $\alpha$  protein,  
15 including SEQ ID NOS: 38 and 39, polynucleotides encoding a Pur $\alpha$ -like protein, including SEQ ID NO: 40, and polynucleotides encoding a hnRNP D protein. Methods for making, introducing and expressing such DNA constructs are well known in the art.

Transcription of the CD95 gene and expression of CD95 may alternatively  
20 be modulated by modulating the activity or function of one or more transcription factors. This may be accomplished, for example, by introducing drugs that interact with the transcription factors to inhibit or activate their binding activity or function.

Modulation of genes other than CD95 according to the methods described above may be accomplished for example, by making a DNA construct in which  
25 coding portions of selected genes are operably linked with a regulatory polynucleotide of the present invention and a suitable promoter. Appropriate transcription factors may be introduced *in vitro* or *in vivo* and play a role in modulating transcription and expression of the selected gene. Similarly, any of the techniques described above for modulating expression of CD95 may be adapted for  
30 modulating expression of other genes operably linked with a regulatory sequence of the present invention. Techniques for synthesizing functional DNA constructs of this type are well known.

Yet another aspect of the present invention relates to the identification of CD95 transcriptional start sites. Several such sites are identified below.

The regulatory polynucleotides and transcription factors of the present invention have numerous uses and applications. Such polynucleotides and transcription factors are useful, for example, for studying regulation of CD95 transcription, and for modulating transcription and expression of coding portions of the CD95 gene, or other genes, as described above, both *in vitro* and *in vivo*.

Regulatory polynucleotides and CD95 transcription factors of the present invention are useful for studying regulation of CD95 both *in vitro* and *in vivo*. For example, regulatory polynucleotides and CD95 transcription factors are useful for identifying cell types and populations having CD95 transcription enhancing and/or silencing regulatory capabilities. Numerous techniques may be employed. Using CD95 regulatory polynucleotides as probes, for example, nuclear extracts from various cell sources may be screened by electrophoretic mobility shift assay (EMSA) for the presence or absence of the respective DNA/polypeptide complexes. Expression of transcription factors capable of binding to such probes can be directly assayed by amplifying a portion of their cDNAs, for example by polymerase chain reaction ("PCR"), or by detecting mRNA for these factors using DNA/RNA or RNA/RNA hybridization techniques, such as Northern analysis or RNase protection assays. The CD95 silencer and enhancer polynucleotides may also be used to screen for the presence and/or activity of the respective regulatory factors in a cell transfection system, wherein expression of various well established reporter genes, such as chloramphenicol acetyl transferase gene, beta galactosidase gene, firefly luciferase gene, renilla luciferase gene, or green fluorescent protein gene is detected. Identification of cell types and populations which contain CD95 regulatory factors may have significant ramifications for the development of therapeutic and prophylactic agents.

Polynucleotides of the present invention also have application for identification of modulators (positive or negative regulators) of CD95 transcription. A screening assay, for example, may utilize transiently or stably transfected reporter constructs comprising the regulatory polynucleotides of the present invention to assess CD95 transcription. As described above, the regulatory silencer and/or

enhancer sequences may be fused to an appropriate promoter driving the expression of one of the above mentioned reporter genes. Such reporter constructs may be transiently transfected, e.g. by lipofection, electroporation, DEAE dextran or Ca-phosphate co-precipitation methods, into appropriate cell lines or primary cells.

5 Reporter activity may then be measured by chemiluminescent, fluorescent, ELISA-based or enzymatic methods (radioactive or non-radioactive). Such screening assays compare favorably with assays that assess the protein turnover of the CD95 receptor.

According to another aspect of the invention, the use of eukaryotic host cells transfected with regulatory polynucleotides of the present invention that mimic the regulated, inducible transcription of the CD95 gene allows identification and testing

10 of the potency of physiological stimulators and inhibitors of CD95 transcription.

Polynucleotides of the present invention are also useful in screening assays to identify molecules capable of binding to regulatory portions of the CD95 gene and thereby regulating transcription of the CD95 gene. Assays for identifying

15 binding molecules using polynucleotide probes are well known in the art and include affinity purification using, for example, trapping of specific DNA/polypeptide complexes formed with biotinylated binding sequences, to a streptavidin matrix or coupling binding site-containing polynucleotides covalently to an appropriate column matrix, such as activated agarose or sepharose. The CD95 regulatory

20 polynucleotides may also be used to monitor specific binding activity of individual fractions of nuclear or whole cell extracts from appropriate sources after treatment by various biochemical and/or biophysical fractionation regimens. The CD95 regulatory polynucleotides may also be employed in a yeast one-hybrid functional cloning system. Regulatory polynucleotides may be cloned in a yeast shuttle vector

25 to activate transcription of a biosynthetic marker or a survival gene which is expressed when at least the DNA binding domain of the cognate transcription factor (provided by a cDNA library and expressed in a second vector as a hybrid with the activation domain of another, suitable transcription factor) binds to this sequence. Identification, isolation and purification of such binding molecules provides a

30 mechanism for modulating transcription of coding portions of the CD95 gene, both *in vitro* and *in vivo*.

Similarly, CD95 transcription factors of the present invention are useful for identification and purification of functionally associated regulatory polypeptides. The transcription factors themselves, or monoclonal antibodies raised against them, may be used for the identification of cell types that contain such regulatory polypeptides. Well known techniques may be employed to raise monoclonal antibodies.

Polynucleotides of the present invention corresponding to regulatory portions of the CD95 gene, or portions of such polynucleotides, may be used as probes to identify and isolate corresponding genomic regions from other species.

10 Identification of such regions aids in identifying structurally conserved motifs which may also exhibit conserved function. Identification of conserved regulatory elements is an important predictive element for extrapolating experimental data from non-human sources to expression of the human CD95 gene.

Regulatory polynucleotides of the present invention are also useful for screening purposes to identify polynucleotides from non-human sources that exhibit homology to the identified sequences. The identification and isolation of CD95 regulatory polynucleotides makes possible the development of transgenic mammalian species having a modified CD95 gene structure lacking a silencer or enhancer regulatory region. Techniques such as homologous recombination and knockout strategies are well known. Such mammalian species are useful as models for studying CD95 gene regulation and apoptosis *in vivo*. Transgenic species with portions of a CD95 promoter or heterologous promoter having regulatory sequences of the present invention fused to a reporter gene may be used, for example, to analyze cross-species regulatory activities of the identified polynucleotide motifs *in vivo*. Transgenic species may also serve as *in vivo* models to screen for tissue specific modulators of CD95 expression. Transgenic species expressing a reporter gene, e.g. beta-galactosidase, which is driven by the CD95 promoter and enhancer, with or without silencer sequences, may also serve as *in vivo* models to screen for tissue specific modulators of CD95 expression. Compounds delivered to such transgenic species can be assayed for their *in vivo* effects on transcription of the reporter gene in the different constructs. In one mouse model of systemic autoimmunity, *lpr* homozygous mice, for example, the key abnormality is defective

expression of the CD95 gene. (Abul K. Abbas, "Die and Let Live: Eliminating Dangerous Lymphocytes," *Cell* 84:655-657, 1996.)

Polynucleotides corresponding to regulatory portions of the CD95 gene and CD95 transcription factors also have numerous therapeutic applications. The CD95 enhancer and silencer polynucleotides may be used, for example, to co-express target/effector genes with native CD95 and thus target cells which can undergo apoptosis. CD95 regulatory polynucleotides cloned in front of the native CD95 gene promoter or a heterologous promoter could be used for regulated co-expression of inhibitors or stimulators of apoptosis in cell types which express CD95, some of which are also susceptible to activation induced cell death. Exemplary inhibitors of CD95 apoptosis are CrmA, a viral inhibitor of the ICE-like cysteine proteases involved in apoptosis, and a dominant negative mutant of the CD95-associated protein FADD. Expression of wildtype FADD can be used to induce apoptosis.

Polynucleotides of the present invention may also be used in gene therapy applications to enhance or silence CD95 expression. A minigene comprising CD95 minimal regulatory sequences which may include, for example, nucleotide positions -1032 to -1 in the hCD95 promoter (which are required for native expression) fused to CD95 cDNA, is useful for enhancing CD95 transcription and expression through gene therapy. Such a minigene may be introduced, for example, in reconstitution and gain-of-function gene therapy in CD95-deficient autoimmune patients. To reconstitute regulated expression of wildtype CD95 in the appropriate cell types of autoimmune patients with a CD95 mutation or expression defect, crude bone marrow cells (or a cell fraction enriched for lymphocytes) from a patient or a compatible donor could be transfected with a CD95 minigene, cloned in viral or non-viral vectors, and these cells reinjected into the patient after destruction of the patient's remaining, untransfected bone marrow cells by radio and-or chemotherapy. Other suitable gene and/or cell therapy approaches are known in the art.

Polynucleotides containing all or a portion of the enhancer or silencer regulatory sequences disclosed herein may also have therapeutic applications as competitors with endogenous binding proteins or transcription factors for regulatory binding sites on the CD95 gene. Suitable delivery techniques are known in the art. CD95 expression may be modulated *in vitro* or *in vivo* using this technique.



Similarly, transcription factors may be employed using competitive or anti-sense strategies to modulate CD95 expression. Degradation-stabilized phosphorthiodate oligonucleotides, containing silencer and/or enhancer sequences, could be encapsulated into liposomes and delivered to patients by injection intravenously or directly into a target site. Alternatively, retroviral or adenoviral vectors, or naked DNA expressing anti-sense RNA for enhancer and/or silencer transcription factors, could be delivered into patient's cells *in vitro* or directly into patients *in vivo* by appropriate routes. Suitable techniques are known in the art.

The word "polynucleotide(s)," as used herein, means a polymeric collection of nucleotides and includes DNA and RNA molecules, both sense and anti-sense strands, and comprehends cDNA, genomic DNA, and wholly or partially synthesized polynucleotides. It will be recognized that operable anti-sense polynucleotides may comprise a fragment of the full-length sequence, and the definition of "polynucleotide" therefore includes all such operable anti-sense fragments. Identification of human genomic DNA and heterologous species DNAs can be accomplished by standard DNA/DNA hybridization techniques, under appropriately stringent conditions, using all or part of a cDNA sequence as a probe to screen an appropriate library. Alternatively, PCR techniques using oligonucleotide primers that are designed based on known genomic DNA, cDNA and protein sequences can be used to amplify and identify genomic and cDNA sequences. Synthetic DNAs corresponding to the identified sequences and variants may be produced by conventional synthesis methods. All of the polynucleotides described herein are isolated and purified.

The word "variant(s)," as used herein in connection with polynucleotides, comprehends polynucleotides having nucleotide sequences different from the specifically identified sequences, wherein one or more of the nucleotides is deleted or substituted, or one or more nucleotides are added, without appreciable loss of the regulatory activity of the identified sequence(s). Polynucleotide variants may be naturally occurring allelic variants, or non-naturally occurring variants. Variant polynucleotides preferably exhibit at least 50%, more preferably at least 70% and most preferably at least 90% identity to the identified regulatory sequences and cDNA sequences. Variant polynucleotides more preferably exhibit at least 70% and

most preferably at least 90% identity to any 8 nucleotide contiguous portion of an identified regulatory sequence and any 50 nucleotide contiguous portion of an identified cDNA sequence. More preferably yet, variant polynucleotides differ from an identified regulatory or cDNA sequence by substitution, deletion or addition of  
5 five nucleotides or fewer. The identity of polynucleotides may be determined by comparing sequences using, for example, algorithms from the FASTA or BLAST search programs (GCG software package, University of Wisconsin) for cDNA comparisons and programs such as TFSEARCH (Yutaka Akiyama, Kyoto University), to search the Transfac database (GBF, Braunschweig, Germany) or  
10 search algorithms used in the TESS database, to compare regulatory sequences.

The word "polypeptide," as used herein, encompasses amino acid chains of any length, including the full length proteins, wherein amino acid residues are linked by covalent peptide bonds. Polypeptides of the present invention may be naturally purified products, or maybe produced partially or wholly using recombinant  
15 techniques. Such polypeptides may be glycosylated with mammalian or other eukaryotic carbohydrates or may be non-glycosylated.

Regulatory polynucleotides and transcription factors are described herein with reference to activities involving "enhancing" or "silencing" transcription of coding portions of the CD95 gene or another gene appropriately linked to a  
20 regulatory polynucleotide sequence and a suitable promoter. Such regulatory activities are observed and may be assessed both *in vitro* and *in vivo*. It will be recognized that organisms and cells of different types, as well as cells in different developmental stages and physiological or *in vitro* conditions, may exhibit substantially different transcriptional activities. Regulatory polynucleotides  
25 described herein as having "enhancing" or "silencing" activities are described with reference to transcription of a CD95 gene having a CD95 basal promoter (-421/-1-CAT) or an HSV tk promoter. Transcriptional activity is considered to be "enhanced" or "silenced" when there is at least a 50%, and more preferably at least a 100%, change in the level of transcriptional activity in the presence of a regulatory  
30 polynucleotide compared to the transcriptional activity measured under substantially the same conditions in the presence of the CD95 basal promoter or the HSV tk promoter. Similarly, transcriptional activity is considered to be "enhanced" or

silenced" when there is at least a 50%, and more preferably at least a 100%, change in the level of transcriptional activity in the presence of a transcription factor or polynucleotide/transcription factor complex compared to the transcriptional activity measured under substantially the same conditions in the absence of a transcription factor or polynucleotide/transcription factor.

Polypeptide transcription factors are described herein with reference to approximate molecular weights. "Approximate" molecular weights contemplate variances of up to 5% of stated molecular weights up to 50 kDa; variances of up to 10% of stated molecular weights from 51 kDa - 100 kDa; and variances of up to 25% of stated molecular weights from 101 - 300 kDa. Binding of polypeptide transcription factors to polynucleotides and formation of DNA/polypeptide complexes may be assessed *in vitro* using standard EMSA techniques described below, or *in vivo* by measuring enhancement or silencing of transcription from the CD95 gene or another gene appropriately linked to a regulatory polynucleotide and a suitable promoter.

The words "isolated" and "purified," and other terms used herein, are used in accordance with their art-recognized meanings.

#### Brief Description of the Drawings

Preferred embodiments of the applicants' invention will be described with reference to the drawings, in which:

Fig. 1 shows results of the functional analysis of the hCD95 gene 5'-flanking region by transient transfection of CAT reporter constructs. The 5'-flanking region of the hCD95 gene is illustrated at the top, with restriction sites relevant for subcloning identified using the following abbreviations: H, *Hind* III; P, *Pst* I; and S, *Sac* II. Individual reporter constructs are illustrated, with construct names referring to nucleotide positions of the subcloned regions of the hCD95 gene. The results of transient transfections into HeLa and COS-7 cells are illustrated in lanes B and C, respectively. These results identify regions in the hCD95 gene that enhance (E1; -1007 to -964) and silence (S1; -1035 to -1008) transcription from the hCD95 promoter.

Fig. 2 illustrates the identification of transcriptional start sites for the hCD95 gene by primer extension analysis on total RNA from Jurkat cells (lane 1), rat lung cells (lane 2) and rat small intestine cells (lane 3). Numbers at the right refer to nucleotide positions upstream of the translational start site in the hCD95 gene.

5 Fig. 3 illustrates the results of electrophoretic mobility shift assay (EMSA) analysis demonstrating that a hexameric inverted repeat sequence identified in SEQ ID NO: 5 (IR2), present in the hCD95 enhancer region, mediates sequence specific binding of transcription factors in Jurkat cell nuclear extract. Distinct DNA/polypeptide complexes are marked by an arrow and arrowhead. Mutational scanning of the hexameric inverted repeat identified in SEQ ID NO: 5, as shown  
10 above the lanes, defined the contributions of individual nucleotide positions to binding and established the degenerate enhancer consensus motif polynucleotide sequence identified in SEQ ID NO: 3.

Fig. 4 illustrates the results of EMSA analysis demonstrating that novel  
15 DNA/polypeptide complexes were formed in a sequence-specific manner. Complexes formed with hCD95 enhancer region motifs spaced by 1 bp and 4 bp are marked by an open arrowhead. This data suggests the existence of a family of related transcription factors which recognize the same binding motif but have different spacing requirements.

20 Fig. 5 illustrates the results of EMSA analysis demonstrating that a novel DNA/polypeptide complex was formed with an hCD95 silencer region probe and an enhancer probe. The experimental work suggested that the polynucleotide heptamer motif identified as SEQ ID NOS: 7 or 36 mediates interaction with transcription factor(s).

25 Fig. 6 illustrates the results of EMSA analysis demonstrating that single-stranded probes compete for complex-formation and interruption of the heptamer motif identified as SEQ ID NO: 7 or 36 in the silencer region abolishes the ability of the probe to compete with wild-type silencer probe for complex formation. The polynucleotide heptamer motif is thus important for regulatory silencing function.  
30 The SEQ ID NOS for the probe sequences are identified above the lanes.

Fig. 7 illustrates the results of UV-crosslinking analysis. Fig. 7A shows UV-crosslinking using nuclear extracts from murine L929 cells with a double-stranded

hCD95 enhancer region probe (SEQ ID NO: 1). Distinct DNA/polypeptide complexes of approximately 59 and 113 kDa, and a high molecular weight complex of approximately 200-300 kDa are identified. Fig. 7B shows the results of UV-crosslinking using nuclear extracts from Jurkat and L929 cells with a single-stranded hCD95 silencer region probe (SEQ ID NO: 2) to identify DNA/polypeptide complexes of approximately 47, 77 and 100 kDa.

Fig. 8 illustrates the results of Southwestern analysis. Fig. 8A shows the results of Southwestern analysis using nuclear extracts from Jurkat and rat dermal papilla (rDP) cells with a double-stranded hCD95 enhancer region probe (SEQ ID NO: 11). Distinct DNA/polypeptide complexes of approximately 113kDa (in Jurkat and rDP) and approximately 59 kDa (in rDP) were identified. Fig. 8B shows the results of Southwestern analysis using nuclear extracts from Jurkat cells with a single stranded silencer region probe (SEQ ID NO: 2). Distinct DNA/polypeptide complexes of approximately 47 kDa and 100 kDa were identified.

Fig. 9 shows results of the transient transfection of CAT reporter constructs including various hCD95 enhancer and silencer region polynucleotides. Individual reporter constructs, including those referred to as IR2-tk-CAT (SEQ ID NO: 11), mIR2-tk-CAT (SEQ ID NO: 19), IR1-tk-CAT (SEQ ID NO: 4), IR4-tk-CAT (SEQ ID NO: 6) and S1-tk-CAT (SEQ ID NO: 2), are illustrated, with construct names referring to the hCD95 enhancer and/or silencer region polynucleotides. The results of transient transfections into HeLa and COS-7 cells are illustrated in lanes B and C, respectively. The hCD95 enhancer polynucleotides autonomously enhance transcription from the heterologous tk promoter only in the absence of the silencer region. These results demonstrate the *in vivo* functionality of the identified hCD95 enhancer and silencer regions.

#### Detailed Description of the Invention

Genomic clones for the human CD95 (hCD95) gene were isolated and a 2.3 kb region of the hCD95 gene 5'-flanking region was sequenced. The hCD95 polynucleotide sequence is assigned accession number X87625 in the EMBL database. Initial functional analysis, using CAT reporter constructs and transient transfections, identified transcription silencer activity residing between nucleotide

positions -1,781 and -1,007 of the hCD95 gene, and strong transcription enhancer activity residing between nucleotide positions -1,007 and -425 of the human CD95 gene. This experimental work is described in F. Rudert et al., "Identification of a Silencer, Enhancer, and Basal Promoter Region in the Human CD95 (Fas/APO-1) Gene," DNA AND CELL BIOLOGY, Vol. 14, No. 11, pp. 931-937, 1995. Additional functional analysis further delineated the enhancer and silencer regions. A transcription enhancer region, denominated E1 (SEQ ID NO: 1), resides between nucleotide positions -1007 and -964 in the hCD95 gene, and a transcription silencer region, denominated S1 (SEQ ID NO: 2), resides between nucleotide positions -1035 and -1008 in the hCD95 gene. These regions mediate cell type-specific and activation state-dependent transcriptional regulation of the CD95 gene during activation-induced cell death.

Further experimental work identified a hexameric inverted repeat binding sequence (IR2) (SEQ ID NO: 5), present in the enhancer region (E1), that mediates sequence specific binding of nuclear factors present in several mammalian cell lines. Contributions of the individual nucleotide positions to binding were assessed and a degenerate enhancer consensus motif binding sequence (SEQ ID NO: 3) was identified. Spacing derivatives of the enhancer region (E1) consensus motif binding sequence (identified in SEQ ID NOS: 4, 6) also formed novel complexes with mammalian nuclear extracts. This data suggests the existence of a family of related transcriptional factors that recognize the same enhancer motif binding sequence, but have different spacing requirements. Enhancer region (E1) binding sequences autonomously enhanced transcription from the heterologous HSV thymidine kinase ("tk") promoter only in the absence and not in the presence of the silencer region, demonstrating the *in vivo* functionality of the regulatory sequence motifs. A heptamer motif binding sequence (SEQ ID NOS: 7 and 36), which is present in identical copies in the hCD95 enhancer and silencer regions, that may mediate binding of nuclear factor(s) to the silencer S1 region, was also identified.

Proteinaceous transcription factors that bind to CD95 regulatory polynucleotides have also been identified. UV cross-linking analysis using an hCD95 silencer probe (SEQ ID NO: 2) showed cross-linked DNA/polypeptide complexes of approximately 47, and 77 and 100 kDa with both human and rodent

nuclear extracts. Results from probing a Southwestern blot of Jurkat cell nuclear extract with a single stranded silencer probe suggested that the 47 and 100 kDa complexes corresponded to single nuclear proteins. Heptamer-containing silencer sequence competitor (SEQ ID NO: 12), complementary to the single stranded silencer probe (SEQ ID NO: 2), but not competitor corresponding to the probe DNA strand (SEQ ID NO: 13) competed for binding of the 47 and 100 kDa species. This correlates with results from EMSA experiments suggesting that the silencer DNA/polypeptide complex is preferably or exclusively formed with single-stranded DNA and that double-strandedness of the DNA at or near the binding region prevents silencer complex formation. The complementary competitor (SEQ ID NO: 12) either contains a silencer binding site (SEQ ID NO: 7) and competes directly with the single-stranded silencer probe (SEQ ID NO: 2) or prevents silencer factor binding by double-strand formation. UV cross-linking analysis using an enhancer probe (SEQ ID NO: 1) and murine cell extract identified cross-linked DNA/polypeptide complexes having molecular weights of about 113 kDa and 59 kDa, and a high molecular weight cross-linked DNA/polypeptide complex of about 200 - 300 kDa.

The present invention is illustrated by reference to the following experimental protocols and results identifying regulatory polynucleotides and transcription factors. The experimental protocols and results support the specification and claims and should not be construed to limit the invention, as claimed, in any fashion.

#### IDENTIFICATION OF HCD95 REGULATORY POLYNUCLEOTIDES

25

##### Isolating and Sequencing Genomic Clones for Human CD95

Clones ( $5 \times 10^5$ ) of a human genomic phage library from placenta were screened with a cDNA probe corresponding to the coding region of hCD95 (EMBL database, accession number x87625).  $\lambda$  phages were grown on *E. coli* Tap90 and replica-plated onto Hybond N<sup>+</sup> nylon filters (Amersham). After denaturation and fixing of the DNA, filters were hybridized with the random-primed probes in 40% formamide, 1 M NaCl, 1% NaDodSO<sub>4</sub>, 10x Denhardt's, 50mM TrisHCl pH 7.5, 2

mM EDTA, 200  $\mu$ g denatured salmon sperm DNA at 42°C overnight. Filters were washed in 2x SSPE/0.1% NaDodSO<sub>4</sub> at 65°C. Positive phage clones were isolated after autoradiography and plaque-purified twice using the CD95 probe. A 3.7-kb *Hind* III fragment from a partial *Hind* III digest of purified phage DNA was identified by Southern analysis using oligonucleotide FR257 (SEQ ID NO: 8) corresponding to positions -205 to -184 in hCD95 cDNA (Itoh et al., "The Polypeptide Encoded by the cDNA for Human Cell Surface Antigen Fas Can Mediate Apoptosis," *Cell* 66:233-243, 1991) and was subcloned in pBS SKII<sup>+</sup> (Stratagene) and partially sequenced. The sequence was determined by PCR cycle sequencing using either <sup>32</sup>P-labeled primers or fluorescent-labeled dideoxynucleotides and a model 373A automated sequencer (Applied Biosystems).

#### Cloning of Initial Human CD95 Gene Reporter Constructs

A panel of 5' deletions of the 5'-flanking region of the human CD95 gene between positions -1781 and -425 was generated by PCR amplification from the genomic hCD95 clone described above. Several reporter constructs were made by cloning selected segments of the hCD95 gene in front of the chloramphenicol acetyl transferase (CAT) gene in the reporter plasmid pBLCAT8<sup>+</sup> (Klein-Hitpass et al., "A 13 bp Palindrome is a Functional Estrogen Responsive Element and Interacts Specifically with Estrogen Receptor," *Nucleic Acids Res.* 16, 647-663, 1988). -1781/-67-tk-CAT was constructed by first ligating the 1.7-kb *Hind* III-*Sac* II fragment into the *Hind* III site of *Hind* III/*Bam* HI-digested pBLCAT8<sup>+</sup> and then filling in and ligating the *Sac* II and *Bam* HI sites. -1007/-1-tk-CAT and -1007/-1-CAT were generated by inserting a *Hind* III/*Bgl* II-digested PCR fragment (Primers FR 283: SEQ ID NO: 9 and FR 290: SEQ ID NO: 10) into *Hind* III/*Bam* HI- and *Hind* III/*Bgl* II-digested pBLCAT8<sup>+</sup>, respectively. -1781/-1-CAT was constructed by cloning the 425-bp *Pst* I-*Bgl* II fragment from -1007/-1-CAT into *Pst* I/*Bgl* II digested -1781/-67-tk-CAT. -1781/-67-CAT was constructed by cutting -1781/-67-tk-CAT with *Pst* I/*Bgl* II, filling in the ends, and religating the vector. -425/-1-CAT was derived from -1007/-1-CAT by digestion with *Hind* III/*Pst* I, filling in the ends, and religation of the remaining vector. The PCR conditions were: 50 pmoles of each primer, 200  $\mu$ M dNTPs each, 2mM MgCl<sub>2</sub>, 10mM TrisHCl pH 8.3, 50 mM KCl,



and 2.5 units of *Taq* polymerase (Boehringer). Amplification was done for 1 min at 94°C, 1 min at 55°C, and 1.5 min at 72°C for 30 cycles.

#### Cloning of Additional Human CD95 Gene Reporter Constructs

- 5 An additional panel of 5' deletions of the 5'-flanking region of the human CD95 gene between positions -1781 to -425 was generated by PCR amplification from the genomic hCD95 clone described above and cloned in front of the CAT gene in the reporter plasmid pBLCAT8<sup>+</sup> which lacked the HSV thymidine kinase (tk) promoter. The following CAT-reporter constructs were cloned: -1781/-1-CAT;
- 10 -1687/-1-CAT; -1513/-1-CAT; -1340/-1-CAT; -1299/-1-CAT; -1261/-1-CAT; -1219/-1-CAT; -1175/-1-CAT; -1115/-1-CAT; -1071/-1-CAT; -1035/-1-CAT; -1007/-1-CAT; -963/-1-CAT; -924/-1-CAT; -874/-1-CAT; -802/-1-CAT; -606/-1-CAT; and -425/-1-CAT.

- 15 Deletion constructs were generated by PCR amplification using the fixed downstream primer identified in SEQ ID NO: 9 with an attached *Bgl* II-site and the following respective upstream primers:

-1687/-1-CAT	SEQ ID NO: 20
-1513/-1-CAT	SEQ ID NO: 21
-1340/-1-CAT	SEQ ID NO: 22
-1299/-1-CAT	SEQ ID NO: 23
-1261/-1-CAT	SEQ ID NO: 24
-1219/-1-CAT	SEQ ID NO: 25
-1175/-1-CAT	SEQ ID NO: 26
-1115/-1-CAT	SEQ ID NO: 27
-1071/-1-CAT	SEQ ID NO: 28
-1035/-1-CAT	SEQ ID NO: 29
-963/-1-CAT	SEQ ID NO: 30
-924/-1-CAT	SEQ ID NO: 31
-874/-1-CAT	SEQ ID NO: 32
-802/-1-CAT	SEQ ID NO: 33
-606/-1-CAT	SEQ ID NO: 34

- 20 containing a *Hind* III-site. *Hind* III/*Bgl* II-digested PCR fragments were gel-purified and cloned into the corresponding sites of pBLCAT8<sup>+</sup>. Construction of -1781/-1-CAT, -1007/-1-CAT and -425/-1-CAT reporter constructs is described above. The additional constructs were generated by ligating double-stranded oligonucleotides

having SEQ ID NOS: 4, 6, 11, 19 or the same sequences with a 5' extension corresponding to SEQ ID NO: 2, most of these oligonucleotides also containing a *Hind* III-compatible 5' overhang, into *Hind* III-digested pBLCAT8+. All constructs were confirmed by sequencing using an automated sequencer (Applied Biosystems).

5

#### Transient Transfection of CAT Reporter Constructs

COS-7 (Cynomologous monkey kidney) and HeLa (human cervix carcinoma) cells were cultured in standard Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% fetal calf serum, penicillin, streptomycin, glutamine, and B-mercaptoethanol (2-ME).  $5 \times 10^5$  cells were seeded in 10-cm plastic culture dishes (Falcon) 24 hr prior to transfection. Cells were transfected by the  $\text{CaPO}_4$  method with 5  $\mu\text{g}$  of initial and additional reporter plasmids described above, 1.5  $\mu\text{g}$  of  $\beta$ -galactosidase ( $\beta$ -Gal) expression vector pCH110 (Pharmacia) as internal control, and 8  $\mu\text{g}$  of pBS (Stratagene) as carrier DNA. At 18 hr after transfection, cells were washed once with DMEM, fresh growth medium was added, and the cells incubated for another 24 hr. Thereafter, cells were harvested in 10mM TrisHCl pH 7.5, 1mM EDTA, 150 mM NaCl; spun down; resuspended in 250 mM TrisHCl pH 7.5, 1mM EDTA, 15% glycerol; and extracts prepared by repeated freeze-thaw cycles. Chloramphenicol acetyl transferase (CAT) assays, normalized for  $\beta$ -Gal expression, were done as described in Zelent et al., "Cloning and Murine  $\alpha$  and  $\beta$  Retinoic Acid Receptors and Novel Receptor  $\gamma$  Predominantly Expressed in Skin," *Nature* 339:715-717, 1989 using 0.5 mM acetylCoA (Boehringer) and 0.2  $\mu\text{Ci}$  [ $^{14}\text{C}$ ]chloramphenicol (sp. act. 54 mCi/mmol, Amersham). The reaction products were separated by thin-layer chromatography in  $\text{CHCl}_3$ /methanol (95:5).

25

#### Functional Analysis of CD95 Gene Sequences

The CAT reporter constructs described above were used to functionally analyze the hCD95 gene 5'-flanking region in transient transfection assays. The percentage CAT conversion and average fold stimulation, compared to pBLCAT8+ lacking the tk promoter (set as 1), are shown below for transfections of the initial reporter constructs into COS-7 and HeLa cells.

30

Table 1

Construct	COS-7		HeLa	
	% CAT Conversion	Fold Stimulation	% CAT Conversion	Fold Stimulation
tk-CAT	2.5 ± 1	6	4.9 ± 4.9	10
-1781/-67-tk-CAT	1.8 ± 0.7	5	5.2 ± 4.6	11
-1007/-1-tk-CAT	12.3 ± 5.2	31	50.7 ± 24.7	102
CAT	0.4 ± 0.1	1	0.5 ± 0.4	1
-1781/-1-CAT	0.6 ± 0.2	2	2.1 ± 0.6	4
-1781/-67-CAT	0.8 ± 0.4	2	4.4 ± 1.9	9
-1007/-1-CAT	7.6 ± 4.1	19	29.7 ± 19.0	60
-425/-1-CAT	3.2 ± 0.4	8	6.6 ± 0.6	13

The largest construct tested (-1781/-1-CAT) showed only very weak activity in COS-7 and HeLa cells. The same construct, with a 67-bp deletion at its 3' end (-1781/-67-CAT) gave practically the same response as -1781/-1-CAT in COS-7 cells and an approximately two-fold increase in HeLa cells. When tested together with the heterologous HSV tk promoter, the same fragment (-1781/-67-tk-CAT) showed no additional transcriptional activity compared to that observed with the tk promoter alone in both COS-7 and HeLa cells. However, a truncation of 764 bp (-1007/-1-CAT) at the 5' end of -1781/-1-CAT increased transcriptional activity 19-fold and 60-fold above background levels in COS-7 and HeLa cells, respectively. A similar upregulation was seen with a tk promoter-containing, identical construct (-1007/-1-tk-CAT) in both cell lines. These data indicated that a silencer is located between positions -1,781 and -1,007 in the human CD95 gene 5'-flanking region. A further truncation of 582 bp at the 5' end of reporter -1007/-1-CAT drastically attenuated the strong activity seen with the construct, but -425/-1-CAT retained a basal promoter activity above that observed with -1781/-1-CAT and -1781/-67-CAT. These results demonstrate the presence of an enhancer between -1,007 and -425 in the hCD95 gene 5'-flanking region and revealed a basal promoter activity in the first 425 bp of the hCD95 regulatory region. Basal promoter activity reached a level comparable to that of the tk promoter. Thus, the hCD95 promoter was regarded as relatively strong.

Functional analysis of the hCD95 gene 5'-flanking region by transient transfection of the additional CAT reporter constructs is illustrated in Fig. 1. Individual reporter constructs are illustrated, with construct names referring to nucleotide positions of the subcloned regions of the hCD95 gene. The results of transient transfections into the HeLa and COS-7 cells are illustrated in lanes B and C, respectively. The thin layer chromatograms show the amount of acetylated <sup>14</sup>C-chloramphenicol substrate as generated by the level of CAT enzyme expressed from the respective reporter constructs, where spot intensity correlates with the level of transcriptional activation. These data further delineate regions in the hCD95 gene which enhance (E1; -1007 to -964) or silence (S1; -1035 to -1008) transcription from the CD95 promoter. The results from transfection analysis indicate that the CD95 silencer represses transcription to levels of about 3-fold less than seen with the CD95 basal promoter (-425/-1-CAT), which has a transcriptional activity similar to that of the HSV tk promoter. The CD95 enhancer increases transcription from the CD95 basal promoter about 2- to 4-fold and from the HSV tk promoter about 5 to 10 fold, depending on the cell type.

Delineation of additional regulatory polynucleotides is described below in connection with the identification of transcription factors.

#### DETERMINATION OF HCD95 TRANSCRIPTIONAL START SITES

##### Primer Extension Analysis

Total RNA from Jurkat cells, rat lung cells and rat small intestine cells was extracted according to Chomczynski and Sacchi, "Single Step Method of RNA Isolation by Acid Guanidinium Thiocyanate-Phenol-Chloroform Extraction," *Anal. Bio-Chem.*, 162:156-159, 1987. All subsequent steps were performed using diethylpyrocarbonate-treated H<sub>2</sub>O. For primer annealing, 10 µg total RNA together with 5 pmole γ-<sup>32</sup>P-labeled primer FR257 (SEQ ID NO: 8, 3 x 10<sup>5</sup> cpm) were incubated for 5 min. at 65°C in hybridization buffer (150 mM KCl, 10 mM TrisHCl pH 8.3, 1 mM EDTA) in a total volume of 15 µl and the mixture then slowly cooled to room temperature for 1.5h. To this, 30 µl of primer extension buffer [30 mM TrisHCl pH 8.3, 15 mM MgCl<sub>2</sub>, 8 mM DTT, 0.22 mg/ml actinomycin D, 220 µM

dNTPs, 200 units MMLV reverse transcriptase (BRL)] were added and reverse transcription was carried out at 42°C for 1 h. Then, 105 µl of RNase digestion buffer [20 µg/ml DNase-free RNaseA (BRL), 100 µg/ml sonicated salmon sperm DNA (Sigma), 100 mM NaCl, 10 mM TrisHCl pH 7.5, 1 mM EDTA] were added, followed by digestion at 37°C for 15 min. 15 µl of 3 M sodium acetate were added, the sample extracted with phenol/CHCl<sub>3</sub> and the DNA was precipitated with ethanol. The extension products were resuspended in formamide loading buffer, heat-denatured and separated on a 6% sequencing gel.

#### 10 Identification of Transcriptional Start Sites

In Jurkat cells, multiple putative transcriptional start sites were identified clustered from -54 to -128 (Fig. 2A,B lane 1) on the hCD95 gene. These matched most of the transcriptional start sites that have been detected in human spleen (Behrmann et al., "Structure of the Human APO-1 Gene," *Eur. J. Immunol.* 24:3057-3062, 1994) using 5' RACE PCR. Virtually the same extension products were obtained with RNA extracted from rat lung (Fig. 2 A,B, lane 2), showing that primer FR257 (SEQ ID NO. 8), which spans the ATG in hCD95, could hybridize to rat CD95 mRNA and suggesting that certain start sites are conserved in human and rat CD95 genes. When RNA from rat small intestine was used, additional extension products, not seen in Jurkat cells or rat lung, were obtained (Fig. 2A,B lane 3). Some of these new, putative start sites lie in very close proximity to hCD95 gene start sites identified in the human T-cell lines CEM-6 and Molt-4 (Cheng et al., "Characterization of the Human Fas Gene," *J. Immunol.* 154:1239-1245, 1995) also using primer extension analysis.

25

#### IDENTIFICATION OF TRANSCRIPTION FACTORS WHICH BIND TO REGULATORY HCD95 POLYNUCLEOTIDES

##### Electrophoretic Mobility Shift Assay (EMSA) Protocol

30 Nuclear extracts were prepared from Jurkat (human T lymphoma cells) and MP-1 (human EBV-transformed B cells) grown under 5% CO<sub>2</sub> in RPMI 1640 medium supplemented with antibiotics and 5% fetal bovine serum, and from HeLa, COS-7, CV-1 (COS-7 derivative) and L929 (murine fibroblast cells) grown under

10% CO<sub>2</sub> in DMEM medium supplemented with antibiotics and 5% fetal bovine serum according to the method of Andrews and Faller, "A Rapid Micropreparation Technique for Extraction of DNA-Binding Proteins from Limiting Numbers of Mammalian Cells," Nucleic Acids Research, Vol. 19, No. 9, 1991. If not indicated otherwise, binding reactions contained 5  $\mu$ g nuclear extract (adjusted to give an equal contribution of 40mM NaCl in the binding reaction), 150 mM (or 100 mM) KCl, 2  $\mu$ g of non-specific competitor DNA (poly[d(l-C)] or poly[d(A-T)], as indicated), 12% glycerol, 12 mM Hepes pH 7.9, 4 mM Tris-HCl pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 20 fmole of [ $\gamma$ -<sup>32</sup>P]ATP-labeled probe (double- or single-stranded, as indicated). The indicated amounts of competitor oligonucleotides were added before addition of the nuclear extract and the reaction incubated for 30 min. at room temperature. Three  $\mu$ l loading buffer (12% glycerol, 12mM Hepes pH 7.9, 4 mM Tris-HCl pH 7.9, 1 mM EDTA, 1 mM dithiothreitol, 0.1 % bromophenol blue) were added, the reactions loaded on pre-run (2 h at 150 V) non-denaturing 4% polyacrylamide gels (acrylamide:bisacrylamide, 30:1). The gels were run in 50 mM Tris-HCl (pH 8.5), 380 mM glycine, 2 mM EDTA at 150 V (constant voltage) with water-cooling. Gels were dried and autoradiographed for 1 to 4 days. This assay, and variants of this assay are referred to herein as the "standard EMSA assay protocol."

#### Identification of Transcription Factors Which Bind to hCD95 Enhancer Region

EMSA analysis using an E1 double-stranded probe (E1 probe, SEQ ID NO: 11) and Jurkat cell nuclear extract revealed that a hexameric inverted repeat nucleotide, identified in SEQ ID NO: 5 and present in E1, mediates sequence-specific binding of nuclear factors. Experimental results are shown in Fig. 3. Distinct DNA/polypeptide complexes formed with these nuclear factors, referred to herein as transcription factors, are marked by an arrow and an arrowhead. Transcription factors that bind to the enhancer region hexameric inverted repeat (IR2) identified as SEQ ID NO: 5 are also present in murine L929 cells and other primate and rodent cells, including HeLa, MP-1, COS-7, and rat dermal papilla (rDP) cells.

Mutational scanning of the enhancer region hexameric inverted repeat identified as SEQ ID NO: 5, using a 50-fold molar excess of double stranded competitor oligonucleotides containing the single nucleotide substitutions indicated above the respective lanes (derivatives of SEQ ID NO: 11) in EMSA analysis together with the wildtype enhancer probe (SEQ ID NO: 1, See Fig. 3) has identified the importance of individual nucleotides for binding and defined the degenerate E1 consensus motif identified in SEQ ID NO: 3 as an hCD95 enhancer region (E1) binding site.

Using nuclear extracts from murine L929 cells in EMSA analysis, sequence-specific formation of novel DNA/polypeptide complexes, which were different from enhancer region binding site IR2 complexes, was demonstrated with enhancer region sequence motifs spaced by 1 bp (IR1; SEQ ID NO: 4) and 4 bp (IR4; SEQ ID NO: 6). Experimental results are shown in Fig. 4. Complexes formed by the nuclear transcription factors that bind to hexameric inverted repeat (SEQ ID NO: 5), containing enhancer probe (IR2, SEQ ID NO: 11), are marked by an arrow and arrowhead. The open arrowhead indicates the presence of complexes formed by the enhancer region spacing derivatives (IR1; SEQ ID NO: 4 and IR4; SEQ ID NO: 6). The enhancer region IR1, IR2 and IR4 elements cross-competed for the formation of the respective DNA/polypeptide complexes in a sequence-specific manner. These results suggest the existence of a family of related transcription factors which recognize the same CD95 enhancer region binding motif but have different spacing requirements.

Concatamerized enhancer (SEQ ID NO: 11) sequences were used as probes to screen cDNA expression libraries according to methods published in Singh et al., (1989) Molecular Cloning of Sequence-Specific DNA Binding Proteins Using Recognition Site Probes, *BioTechniques*, 7 (3), pp 252-261. Using a single-stranded 3x E1 (3x SEQ ID NO: 11) probe to screen a HeLa expression library, clones encoding the following DNA-binding proteins were isolated: human YB-1 (EMBL Accession No. M24070; SWISS-PROT Accession No. P16990); and human hnRNP D (EMBL Accession No. D55672; SWISS-PROT Accession Nos. Q14101 and Q14103).

### Identification of Transcription Factors which Binds to the hCD95 Silencer Region

Novel DNA/polypeptide complexes were formed using a silencer region probe (SEQ ID NO: 2), and an enhancer region IR2 probe (SEQ ID NO: 11) when using polydAdT instead of polydIdC as non-specific competitor DNA in EMSA analysis. Results are shown in Fig. 5, with the silencer probe identification shown above the lanes and the double arrowhead indicating the novel complex. This factor(s) also bound to single-stranded silencer probes or was competed out by single-stranded silencer and enhancer probes.

Further EMSA analysis is illustrated in Fig. 6, using silencer probes having SEQ ID NOS: 2 and 14-18. The silencer region heptamer motif (SEQ ID NO: 7 or 36), which is present in identical copies in the S1 and E1 regions, appeared to mediate interaction with the transcription factor(s), since the respective DNA/polypeptide complex was also formed with a single-stranded silencer region probe (SEQ ID NO: 12) including the silencer region heptamer sequence, but not with the complement of this probe (SEQ ID NO: 13). Silencer region probes from both DNA strands, having an interruption of the heptamer motif in the silencer region and containing less than a full heptamer motif, identified in SEQ ID NOS: 15-18, showed greatly reduced ability to compete for complex formation with the wildtype S1 probe.

Concatamerized silencer (SEQ ID NO: 2) sequences were used as probes to screen cDNA expression libraries according to methods published in Singh et al., (1989) Molecular Cloning of Sequence-Specific DNA Binding Proteins Using Recognition Site Probes, *BioTechniques*, 7 (3), pp 252-261. Using a single-stranded 3XS1 (3x SEQ ID NO: 2) probe to screen HeLa and rat Dermal Papilla expression libraries, clones encoding the following DNA-binding proteins were isolated: human YB-1 (EMBL Accession No. M24070; SWISS-PROT Accession No. P16990); rat YB-1 (EMBL Accession No. M57299; SWISS-PROT Accession No. P22568); rat Pur $\alpha$  (SEQ ID NOS: 38, 39, 41 and 42); and a novel rat Pur $\alpha$ -like protein (SEQ ID NOS: 40 and 43).

To confirm that YB-1 is a component of the CD95 Silencer complex, a YB-1 antibody was incubated with nuclear extracts and the silencer regulatory sequence (SEQ ID NO: 2) probe using supershift procedures documented in Macdonald et al.



(1995), The transcriptional regulatory protein, YB-1, promotes single-stranded regions in the DRA promoter, *J. Biol. Chem.*, 270 (8): 3527-3533. Incubation with the antibody caused a supershift, indicating that YB-1 is a component of the silencer complex.

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Characterization of Transcription Factors for Silencer/Enhancer Regions by UV-crosslinking

UV-crosslinking was performed essentially as described by Miyamoto et al., *Methods Enzymol.* 254, 633-641, 1995. Oligonucleotides of 44- and 28- bases in length were end-labeled as in the above-described EMSA reactions. Double-stranded DNA probes were prepared by annealing the end-labeled oligonucleotides and filling in with [ $\gamma$ - $^{32}$ P]dATP, [ $\gamma$ - $^{32}$ P]dCTP, [ $\gamma$ - $^{32}$ P]dGTP (800Ci/mmol) and 5-bromo-2'-dUTP using Klenow (Miyamoto et al., 1995.)

A standard EMSA binding reaction was set up with 40fmol probe and 10 $\mu$ g nuclear extracts +/- 4pmol competitor DNA in a total volume of 40ml in a flat-bottomed microtitre plate. The plate was covered with Saran-wrap and placed on ice. The reactions were irradiated for 60 minutes by inverting a UV transilluminator of 305nm wavelength, such that the illuminator was within 5cm from the microtitre plate. The reactions were then divided into two. One aliquot was run on a 4% non-denaturing gel as described previously, and the second aliquot was run on a 10% reducing SDS-PAGE gel with  $^{14}$ C-labeled protein markers. The gels were dried followed by autoradiography with an intensifying screen for 1-3 days.

UV-crosslinking analysis results shown in Fig. 7B using an end-labeled, single-stranded S1 probe (SEQ ID NO: 2), revealed cross-linked DNA/polypeptide complexes of approximately 47, 77 and 100 kDa in Jurkat and L929 cells. Results from probing a Southwestern blot of Jurkat cell nuclear extract with the single-stranded S1 probe (SEQ ID NO: 2) suggested that the 47 kDa and 100 kDa complexes corresponded to single nuclear proteins. UV-crosslinking with a double-stranded E1 probe (SEQ ID NO: 1), shown in Fig. 7A, revealed cross-linked DNA/polypeptide complexes of approximately 59 kDa, 113 kDa, and a high molecular weight complex of approximately 200 to 300 kDa in L929 cells.

Characterization of Transcription Factors for Silencer/Enhancer Regions by Southwestern Analysis

20-40 $\mu$ gs of nuclear extracts from Jurkat, L929 and rat dermal papilla (rDP) cells, prepared as described above, were electrophoresed on a 8-10% reducing SDS-PAGE gel with  $^{14}$ C-labeled protein markers. The gel was pre-soaked in Transfer Buffer prior to electroblotting to 0.2mm nitrocellulose filters as described by Li, M. and Desiderio, S., Appendix 1, "Transcription Factors: A Practical Approach" (D.S. Latchman, Ed.) IRL Press, Oxford, pp. 187-196, 1993. Nitrocellulose filters were blocked in 2.5% (w/v) dried milk powder, 25mM Hepes (pH 8), 1mM DTT, 10% (v/v) glycerol, 50mM NaCl, 1mM EDTA at 4°C for 18 hours. Filters were hybridised in SW-Binding Buffer (12% (v/v) glycerol, 12mM Hepes (pH 8), 4mM Tris-HCl (pH 8), 1mM EDTA, 1mM DTT, 40mM NaCl, 100mM KCl), 1pmol/ml  $^{32}$ P-labeled DNA probe (end labeled or filled in, as above), 10 $\mu$ g/ml non-specific competitor DNA (i.e. poly[dl-dC] or poly[dA-dT]) and +/- 100 pmol/ml competitor DNA for 60 minutes at room temperature. The filters were washed for 4 x 7 minutes in SW-Binding Buffer at 4°C, prior to autoradiography for 3 days with an intensifying screen.

Southwestern analysis results of Jurkat and rDP nuclear extracts using a double stranded enhancer probe (SEQ ID NO: 11) are illustrated in Fig 8A. These results show protein species having molecular weights of approximately 59 kDa (rDP) and 113 kDa (Jurkat and rDP) to which a double-stranded enhancer probe binds. Southwestern analysis results of Jurkat nuclear extracts using a single stranded silencer probe (SEQ ID NO: 2) are illustrated in Fig. 8B. These results show protein species having molecular weights of approximately 47 kDa and 100 kDa to which a single-stranded silencer probe binds. Binding of the silencer probe to these proteins is greatly reduced or absent in the presence of a heptamer - (SEQ ID NO: 7) containing competitor (SEQ ID NO: 12), complementary to the probe strand, but not an equivalent competitor (SEQ ID NO: 13) corresponding to the probe strand. These Southwestern results are consistent with UV-crosslinking and EMSA results obtained with the probes and competitors described above.

### Functional Analysis of Transcription Factors

The CD95 silencer (SEQ ID NO: 2), enhancer (SEQ ID NO: 11) and promoter fragment (SEQ ID NO: 27) were cloned in front of the HSV tk promoter and CAT gene in the reporter plasmid pBLCAT8<sup>+</sup>. Constructs encoding YB-1, Pur $\alpha$ , Pur $\alpha$ -like and hnRNP D proteins were cloned into the vector pCDNA3. The constructs were overexpressed in HeLa cells with both of the CD95 promoter-CAT reporter constructs. The overexpression of sense YB-1 repressed transcription of the CD95 promoter 7-fold and expression of antisense YB-1 stimulated transcription of the CD-95 promoter 2-fold. The overexpression of sense Pur $\alpha$  repressed transcription of the CD95 promoter 4-fold and expression of antisense Pur $\alpha$  stimulated the transcription of the CD95 promoter 1.5-fold. This suggests that both YB-1 and Pur $\alpha$  play a role in repression of the CD95 promoter *in vivo*.

A construct encoding hnRNP D protein was also cloned into the vector pCDNA3. This construct was overexpressed in HeLa cells with the CD95 promoter-CAT reporter constructs described above.

### EMSA Analysis of Silencer/DNA and Enhancer/DNA Complexes

EMSA gel mobility shift assays using the protocol described above determined several characteristics of the silencer/DNA (SEQ ID NO: 2) and enhancer/DNA (SEQ ID NO: 11) complexes. The half-life of both silencer/DNA and enhancer/DNA complexes is approximately one hour. The enhancer/DNA complex was only stable in less than 300mM KCl, and formation of the enhancer/DNA complex required the presence of divalent cations. Thirty percent (30%) of the silencer/DNA complex was stable in 2M KCl. The formation of the silencer/DNA complex did not require the presence of divalent cations, and the silencer/DNA complex contains an ATP-dependent protein.

These experimental results demonstrate that the silencer/DNA complex is very stable, and the enhancer/DNA complex is much less stable. This is consistent with the anticipated *in vivo* activity – i.e. the silencer/DNA complex is formed most of the time, and expression of CD95 is suppressed. The ATP-dependence suggests that ATP is utilized to unwind the CD95 silencer regulatory region to provide the single stranded conformation that the transcription factors bind.

DEMONSTRATION OF PROMOTER CONTEXT-INDEPENDENT FUNCTION  
OF hCD95 SILENCER AND ENHANCER REGIONS

CD95 enhancer binding site sequences IR1 (SEQ ID NO: 4), IR2 (SEQ ID NO: 11), mutated IR2 (SEQ ID NO: 19), and IR4 (SEQ ID NO: 6) were cloned, with and without an upstream silencer region (SEQ ID NO: 2), in front of the HSV tk promoter and CAT gene in reporter plasmid pBLCAT8+. The S1/mIR2-tk-CAT construct had an additional base in the mIR2 region and is set forth in SEQ ID NO: 35. The reporter constructs and results of transient transfection assays of these CAT reporter constructs into HeLa (B) and COS-7 (C) cells are shown in Fig. 9. The experimental results show that enhancer region elements in IR1, IR2 and IR4 autonomously enhanced transcription, to various extents, from the heterologous promoter only in the absence, and not in the presence, of silencer region polynucleotides. This demonstrates the *in vivo* functionality of the identified silencer and enhancer polynucleotide sequences.

All references and other materials cited herein are incorporated by reference in their entirety. While in the foregoing specification this invention has been described in relation to certain preferred embodiments thereof, and many details have been set forth for purposes of illustration, it will be apparent to those skilled in the art that the invention is susceptible to additional embodiments and that certain of the details described herein may be varied considerably without departing from the basic principles of the invention.

## SEQUENCE LISTING

## (1) GENERAL INFORMATION

- (i) APPLICANT:
- (ii) TITLE OF THE INVENTION: CD95 REGULATORY GENE SEQUENCES  
AND TRANSCRIPTION FACTORS
- (iii) NUMBER OF SEQUENCES: 43

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- (A) MEDIUM TYPE: Diskette  
(B) COMPUTER: IBM Compatible  
(C) OPERATING SYSTEM: DOS  
(D) SOFTWARE: Wordperfect 5.2

## (vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER:  
(B) FILING DATE:  
(C) CLASSIFICATION:

## (vii) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER:  
(B) FILING DATE:

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## (2) INFORMATION FOR SEQ ID NO:1:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 44 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Other

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

AGTAATGATG TCATTATCCA AACATACCTT CTGTAAAATT CATG

## (2) INFORMATION FOR SEQ ID NO:2:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

GTCTGGAAGT GCATCCAAAT TCAGGTTC  
28

## (2) INFORMATION FOR SEQ ID NO:3:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 13 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

KMMTGAKGTM AKM  
13

## (2) INFORMATION FOR SEQ ID NO:4:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

AGTAATGAT TCATTATCCA AA  
21

## (2) INFORMATION FOR SEQ ID NO:5:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 14 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TAATGATGTC ATTA  
14

## (2) INFORMATION FOR SEQ ID NO:6:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 24 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single

- (D) TOPOLOGY: linear
- (ii) MOLECULE TYPE: Other
- (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

AGTAATGAT GTGTCATTAT CCAAA  
24

(2) INFORMATION FOR SEQ ID NO:7:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 7 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

TTTGGAT  
7

(2) INFORMATION FOR SEQ ID NO:8:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 21 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

TGCCCAGCAT GGTGTTGAG C  
21

(2) INFORMATION FOR SEQ ID NO:9:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 26 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CAAGATCTGG TTGTTGAGCA ATCCTC  
26

(2) INFORMATION FOR SEQ ID NO:10:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 29 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCGAAGCTTA GTAAATGATG TCATTATCC  
29

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 22 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

AGTAATGAT GTCATTATCC AAA  
22

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

GAATTTGGAT GCAG  
14

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CTGCATCCAA ATTC  
14

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

GAACCTGAAT TTGGATGCAG TTCCAGAC  
28

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:



- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GTCTGGAAC GCAT

14

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ATGCAGTTCC AGAC

14

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

CCAAATTCAG GTTC

14

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GAACCTGAAT TTGG

14

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

AGTATGATGG CATTATCCAA A  
21

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

CACATATGTG AGTTGCTGGC  
20

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GCGAAGCTTC TTTTCATTTT GGAATAG  
27

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 28 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

GCGAAGCTTA GGTGGAACAG AGACAAGC  
28

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 25 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GCGAAGCTTT GGTAAGTGCA GTGAC  
25

## (2) INFORMATION FOR SEQ ID NO:24:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Other

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

GCGAAGCTTG AAAGCCCTCA GGAGG  
25

## (2) INFORMATION FOR SEQ ID NO:25:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Other

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

GCGAAGCTTA AACAGGCTCC AGAAG  
25

## (2) INFORMATION FOR SEQ ID NO:26:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Other

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

GATGTACAGT GGGCTAAGC  
19

## (2) INFORMATION FOR SEQ ID NO:27:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 27 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Other

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

GCGAAGCTTG GAAGGGAGAG AGGTTGC  
27

## (2) INFORMATION FOR SEQ ID NO:28:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 25 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:28:

GCGAAGCTTG ATGCCAAAGG AATAC  
25

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GCGAAGCTTG TCTGGAAGT CATCC  
25

(2) INFORMATION FOR SEQ ID NO:30:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

GCGAAGCTTC TAACTACCT AAGAG  
25

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

GCGAAGCTTG TGACTTTGAA CAGTG  
25

(2) INFORMATION FOR SEQ ID NO:32:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 25 base pairs

(B) TYPE: nucleic acid

(C) STRANDEDNESS: single

(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GCGAAGCTTT TTAAAGAAAA TTGGC  
25

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 26 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GCGAAGCTTG GGCTATGCGA TTTGGC  
26

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

GCGAAGCTTC TTTCTCTGAG TGACTCC  
27

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 50 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

GTCTGGAAGT GCATCCAAAT TCAGGTTTCTAG TAATGATGGC ATTATCCAAA  
50

(2) INFORMATION FOR SEQ ID NO:36:

- (i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 7 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

ATCCAAA  
7

(2) INFORMATION FOR SEQ ID NO:37:

- (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 16 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

AGTAATGATG TCATTA  
16

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 678 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

GGGCGACTGA AGCGGCGGGC GGAACGGCAG GCGGCGGCGG ATCGCAGCAT CATGGCGGAC  
60  
CGAGACAGCG GCAGCGAGCA GGGTGGTGCG GCGCTGGGCT CGGGCGGCTC CCTAGGGCAC  
120  
CCGGGCTCGG GCTCAGGCTC CGGCGGGGGC GGTGGTGGCG GCGGGGGCGG CGGCGGCAGT  
180  
GGCGGCGGCG GCGGGGCCCC GGGGGGGCTG CAGCACGAGA CGCAGGAGCT GGCCTCCAAG  
240  
CGGGTGGACA TCCAGAACAA GCGTTTCTAC CTGGACGTGA AGCAGAACGC TAAGGGCCGT  
300  
TTCCTGAAGA TCGCTGAGGT GGGCGCTGGC GGCAACAAGA GCCGCCTCAC CCTCTCCATG  
360  
TCTGTGGCCG TGGAGTTCGG CGACTACCTG GGCGACTTCA TCGAGCACTA CGCGCAGCTG  
420  
GGCCCCAGCC AGCCGCCCCA CCTGGCCCAG GCACAGGACG AGCCACGCCG GGCGCTCAAG  
480  
AGCGAGTTCC TGGTGCGCGA AAACCGCAAG TACTACATGG ATCTCAAGGA GAACCAGCGC  
540  
GGCCGCTTCC TGCGCATCCG CCAGACAGTC AACCGGGGGC CCGGCCTGGG CTCCACGCAG  
600  
GGCCAGACCA TTGCGCTGCC CGCACAGGGG CTCATCGAGT TCCGTGACGC TCTGGCCAAG  
660  
CTCATCGACG ACTATGGA  
678

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 434 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: Other

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

TTCTTCGATG TGGGTTCCTA CAAGTACGGC GTGTTTATGC GAGTCAGTGA GGTGAAGCCC  
60  
ACCTACCGCA ACTCCATCAC CGTGCCCTAC AAGGTGTGGG CCAAGTTCGG ACACACCTTC  
120

TGCAAGTACT CCGAGGAGAT GAAGAAGATT CAAGAGAAGC AGAGGGAGAA GCGGGCCGCC  
 180  
 TGTGAGCAGC TCCACCAGCA GCAACAGCAG CAGCAAGAGG AGACCACCGC TGCCACCCTG  
 240  
 TTATTGCAGG GTGAGGAAGA AGGGGAAGAA GATTGATCAA ACTGAATGAA ACACACACAC  
 300  
 ACACACACAC ACACACACAC ACACACACAC ACGCATACAC ATACGTGTAC ACACACACAC  
 360  
 ACACACACAG CCACACACAG AGAAAATATA CTGTAAAGAG AGAAAATAAA AAGTTAAAAA  
 420  
 AAAAAAAAAA AAAA  
 434

## (2) INFORMATION FOR SEQ ID NO:40:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 384 base pairs  
 (B) TYPE: nucleic acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: Other

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

GAATTCGGCA CGAGCTCGTG CCGAATTCGG CACGAGGGGC GCGAGCGCAG CGGGCGGCGG  
 60  
 CGAGGCGGCG GCCGAGAAGA TGGCGGACGG CGACAGCGGC AGCGAGCGCG GTGGCGGCGG  
 120  
 CGGCGGGCCC GGCAGCTTCC AGCCCGCGCC CCGCGGCGGC GGAGGGCCTG GCGGCGAGCA  
 180  
 GGAGACGCAG GAGCTGGCCT CGAAGCGGCT GGACATCCAG AACAGCGCT TCTACCTGGA  
 240  
 CGTGAAGCAG AACGCCAAGG GCCGCTTCT CAAAATCGCC GAGGTGGGCG CGGGCGGCTC  
 300  
 CAAGAGCCGC CTCACGCTCT CGATGGCGGT GGCCGCCGAG TTCCGCGACT CGCTGGGCGA  
 360  
 CTTTCATCGAG CACTACGCGC AGCT  
 384

## (2) INFORMATION FOR SEQ ID NO:41:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 209 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

Met Ala Asp Arg Asp Ser Gly Ser Glu Gln Gly Gly Ala Ala Leu Gly  
 1 5 10 15  
 Ser Gly Gly Ser Leu Gly His Pro Gly Ser Gly Ser Gly Ser Gly Gly  
 20 25 30  
 Gly Gly Gly Gly Gly Gly Gly Gly Gly Ser Gly Gly Gly Gly Gly  
 35 40 45  
 Ala Pro Gly Gly Leu Gln His Glu Thr Gln Glu Leu Ala Ser Lys Arg  
 50 55 60  
 Val Asp Ile Gln Asn Lys Arg Phe Tyr Leu Asp Val Lys Gln Asn Ala  
 65 70 75 80  
 Lys Gly Arg Phe Leu Lys Ile Ala Glu Val Gly Ala Gly Gly Asn Lys  
 85 90 95  
 Ser Arg Leu Thr Leu Ser Met Ser Val Ala Val Glu Phe Arg Asp Tyr  
 100 105 110  
 Leu Gly Asp Phe Ile Glu His Tyr Ala Gln Leu Gly Pro Ser Gln Pro  
 45

115 120 125  
 Pro Asp Leu Ala Gln Ala Gln Asp Glu Pro Arg Arg Ala Leu Lys Ser  
 130 135 140  
 Glu Phe Leu Val Arg Glu Asn Arg Lys Tyr Tyr Met Asp Leu Lys Glu  
 145 150 155 160  
 Asn Gln Arg Gly Arg Phe Leu Arg Ile Arg Gln Thr Val Asn Arg Gly  
 165 170 175  
 Pro Gly Leu Gly Ser Thr Gln Gly Gln Thr Ile Ala Leu Pro Ala Gln  
 180 185 190  
 Gly Leu Ile Glu Phe Arg Asp Ala Leu Ala Lys Leu Ile Asp Asp Tyr  
 195 200 205  
 Gly

## (2) INFORMATION FOR SEQ ID NO:42:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 91 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

Phe Phe Asp Val Gly Ser Asn Lys Tyr Gly Val Phe Met Arg Val Ser  
 1 5 10 15  
 Glu Val Lys Pro Thr Tyr Arg Asn Ser Ile Thr Val Pro Tyr Lys Val  
 20 25 30  
 Trp Ala Lys Phe Gly His Thr Phe Cys Lys Tyr Ser Glu Glu Met Lys  
 35 40 45  
 Lys Ile Gln Glu Lys Gln Arg Glu Lys Arg Ala Ala Cys Glu Gln Leu  
 50 55 60  
 His Gln Gln Gln Gln Gln Gln Glu Glu Thr Thr Ala Ala Thr Leu  
 65 70 75 80  
 Leu Leu Gln Gly Glu Glu Glu Gly Glu Glu Asp  
 85 90

## (2) INFORMATION FOR SEQ ID NO:43:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 101 amino acids  
 (B) TYPE: amino acid  
 (C) STRANDEDNESS: single  
 (D) TOPOLOGY: linear

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

Met Ala Asp Gly Asp Ser Gly Ser Glu Arg Gly Gly Gly Gly Gly Gly  
 1 5 10 15  
 Pro Gly Ser Phe Gln Pro Ala Pro Arg Gly Gly Gly Gly Pro Gly Gly  
 20 25 30  
 Glu Gln Glu Thr Gln Glu Leu Ala Ser Lys Arg Leu Asp Ile Gln Asn  
 35 40 45  
 Lys Arg Phe Tyr Leu Asp Val Lys Gln Asn Ala Lys Gly Arg Phe Leu  
 50 55 60  
 Lys Ile Ala Glu Val Gly Ala Gly Gly Ser Lys Ser Arg Leu Thr Leu  
 65 70 75 80  
 Ser Met Ala Val Ala Ala Glu Phe Arg Asp Ser Leu Gly Asp Phe Ile  
 85 90 95  
 Glu His Tyr Ala Gln  
 100



We Claim:

1. An isolated polynucleotide selected from the group consisting of:  
SEQ ID NOS: 1-6, 11, 12, 14, 36 and 37.
2. A polynucleotide having at least 70% identity to a polynucleotide of claim 1.
3. A polynucleotide of claim 2 which is a cDNA molecule.
4. A polynucleotide of claim 2 which is a genomic DNA molecule.
5. A polynucleotide of claim 2 which is a wholly or partially chemically synthesized polynucleotide.
6. A polynucleotide of claim 2 in an antisense orientation.
7. A polynucleotide of claim 2 that is capable of binding to a polypeptide transcription factor to form a complex that modulates transcription of the CD95 gene.
8. A polynucleotide comprising a DNA sequence having at least 90% identity to any 8 nucleotide contiguous portion of a polynucleotide of claim 1.
9. A polynucleotide that differs from a polynucleotide of claim 1 by substitution, deletion or addition of five nucleotides or fewer.
10. An isolated polypeptide transcription factor capable of forming a DNA/polypeptide complex with a polynucleotide of claim 2.
11. An isolated polynucleotide capable of forming a DNA/polypeptide complex with a transcription factor, whereby the DNA/polypeptide complex is capable of modulating transcription of a coding portion of the CD95 gene.
12. An isolated polynucleotide according to claim 11 capable of forming a DNA/polypeptide complex having a molecular weight of approximately 47, 77 or 100 kDa with a transcription factor, whereby the DNA/polypeptide complex is capable of silencing transcription of a coding portion of the CD95 gene.
13. An isolated polynucleotide according to claim 11 capable of forming a DNA/polypeptide complex having a molecular weight of approximately 59, 113 or 200-300 kDa with a transcription factor, whereby the DNA/polypeptide complex is capable of enhancing transcription of a coding portion of the CD95 gene.

14. An isolated transcription factor capable of forming a DNA/polypeptide complex with a polynucleotide, whereby the DNA/polypeptide complex modulates transcription of coding portions of the CD95 gene.
15. An isolated transcription factor according to claim 14 capable of forming a DNA/polypeptide complex having a molecular weight of approximately 47, 77 or 100 kDa with a polynucleotide, whereby the DNA/polypeptide complex is capable of silencing transcription of a coding portion of the CD95 gene.
16. An isolated transcription factor according to claim 14 capable of forming a DNA/polypeptide complex having a molecular weight of approximately 59, 113 or 200-300 kDa with a polynucleotide, whereby the DNA/polypeptide complex is capable of enhancing transcription of a coding portion of the CD95 gene.
17. An isolated polynucleotide selected from the group consisting of:  
SEQ ID NOS: 38, 39 and 40.
18. A polynucleotide having at least 70% identity to a polynucleotide of claim 17.
19. A polypeptide comprising an amino acid sequence identified in SEQ ID NOS: 41, 42 or 43.
20. An isolated polynucleotide comprising a DNA sequence of claim 2 or 18.
21. A method for modulating apoptotic cell death comprising regulating the binding of a polypeptide, the polypeptide having a cold shock domain having at least 95% identity to the cold shock domain of human YB-1 (EMBL Accession No. M24070; SWISS-PROT Accession No. P16990), to a polynucleotide of claim 2.
22. A method for modulating apoptotic cell death comprising regulating the binding of a polypeptide, the polypeptide having at least 95% identity to human Pur $\alpha$  (EMBL Accession No. M96684; SWISS-PROT Accession No. Q00577), to a polynucleotide of claim 2.
23. A method for modulating apoptotic cell death comprising regulating the binding of a polypeptide, the polypeptide having at least 95% identity to a polypeptide encoded by a polynucleotide of claim 18, to a polynucleotide of claim 2.
24. A method for modulating apoptotic cell death comprising regulating the binding of a polypeptide, the polypeptide having at least 95% identity to human

hnRNP D (EMBL Accession No. D55672; SWISS-PROT Accession Nos. Q14101 and Q14103), to a polynucleotide of claim 2.

25. A method for modulating CD95 expression by regulating the binding of a polypeptide, the polypeptide having a cold shock domain having at least 95% identity to the cold shock domain of human YB-1 (EMBL Accession No. M24070; SWISS-PROT Accession No. P16990), to a polynucleotide of claim 2.

26. A method for modulating CD95 expression by regulating the binding of a polypeptide, the polypeptide having at least 95% identity to human Pura (EMBL Accession No. M96684; SWISS-PROT Accession No. Q00577), to a polynucleotide of claim 2.

27. A method for modulating CD95 expression by regulating the binding of a polypeptide, the polypeptide having at least 95% identity to a polypeptide encoded by a polynucleotide of claim 18, to a polynucleotide of claim 2.

28. A method for modulating CD95 expression by regulating the binding of a polypeptide, the polypeptide having at least 95% identity to human hnRNP D (EMBL Accession No. D55672; SWISS-PROT Accession Nos. Q14101 and Q14103), to a polynucleotide of claim 2.

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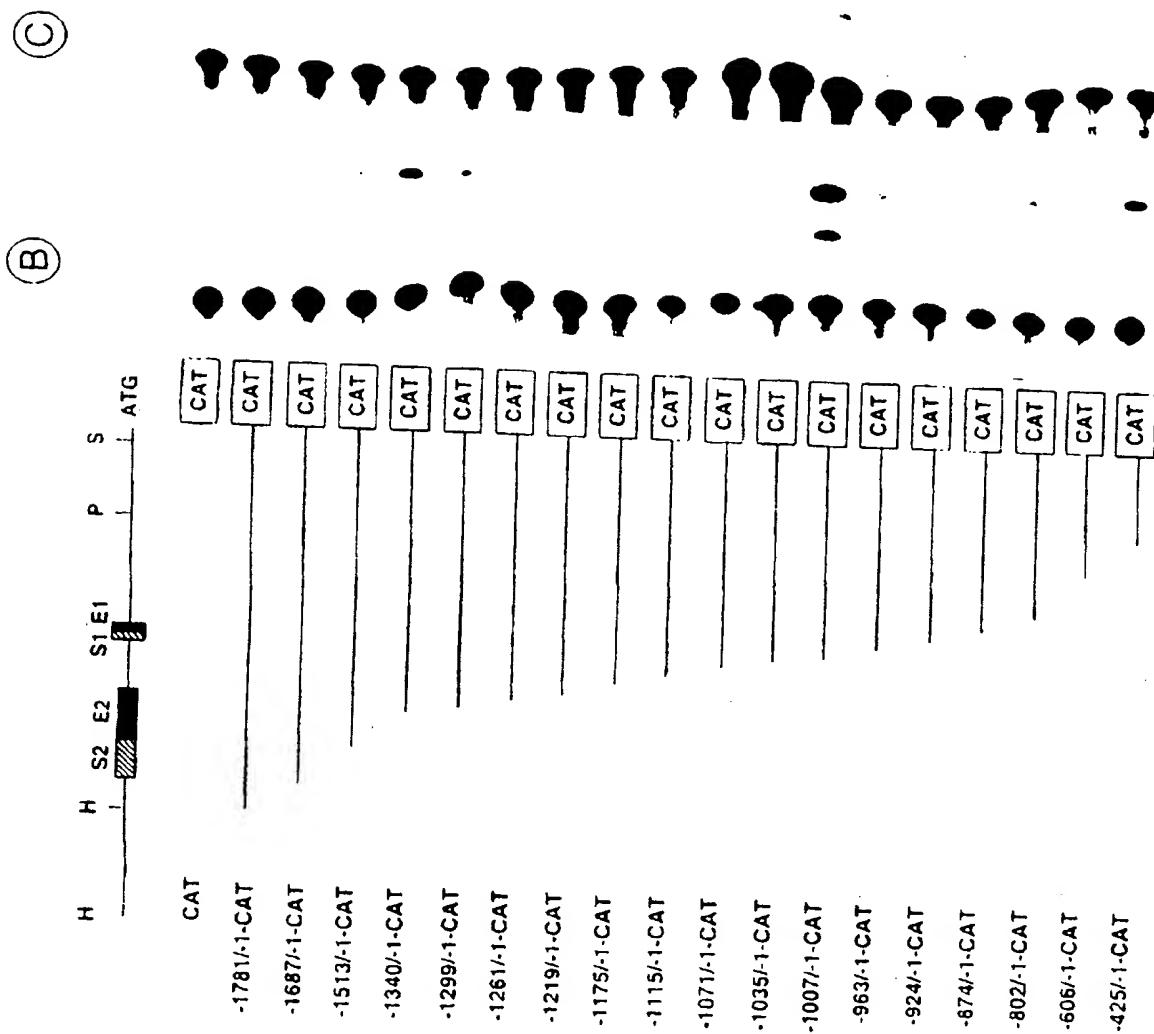


Fig. 1

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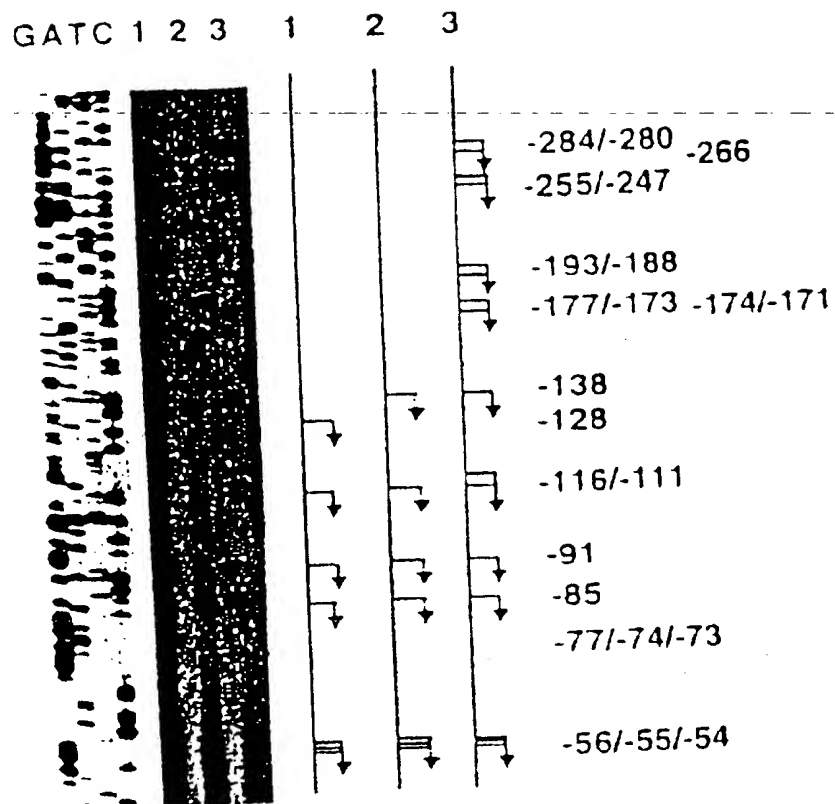
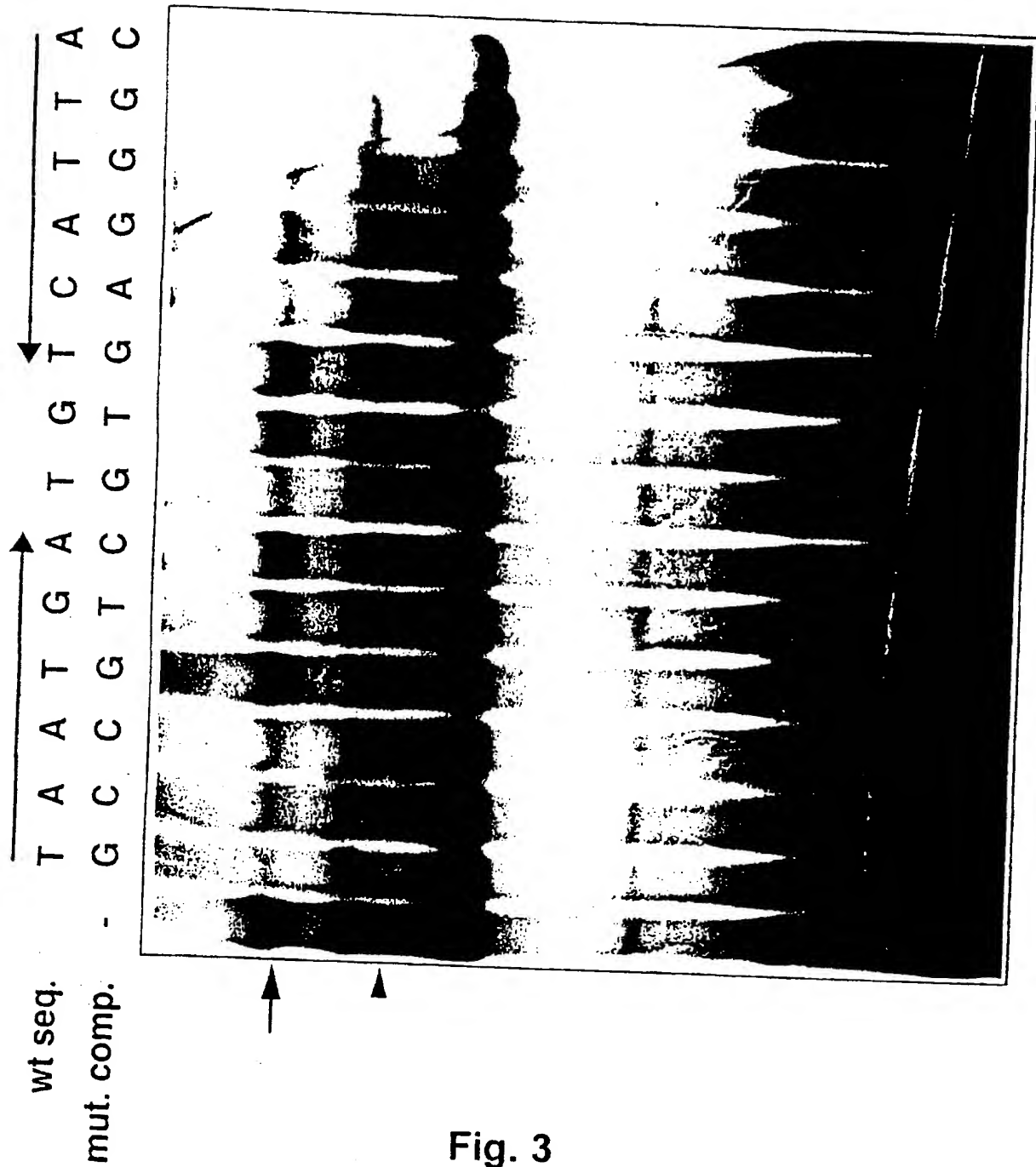


Fig. 2

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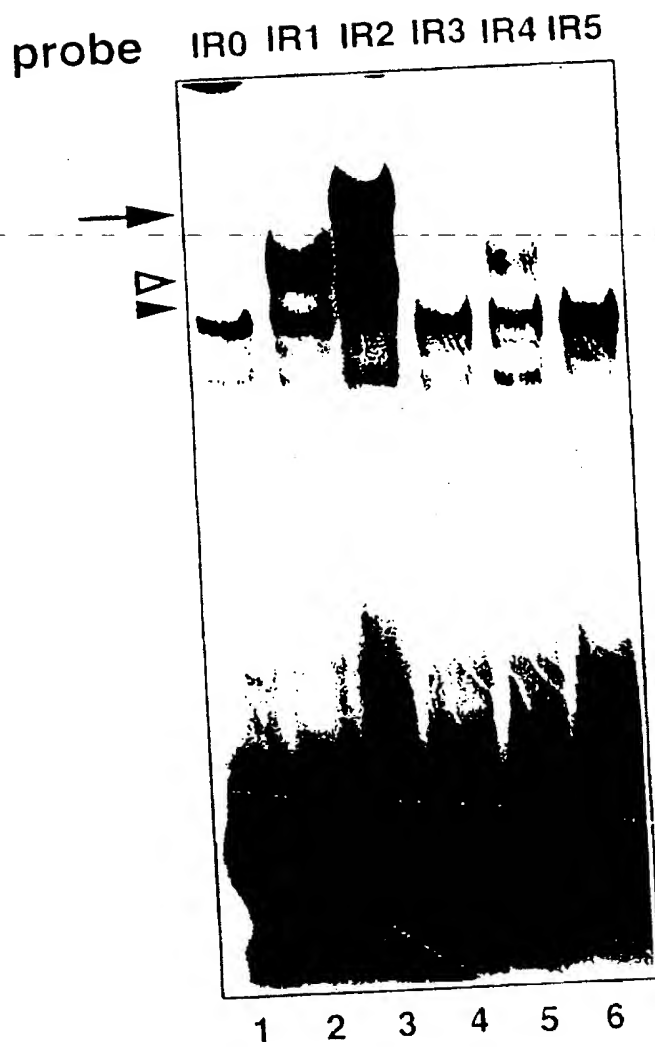


Fig. 4

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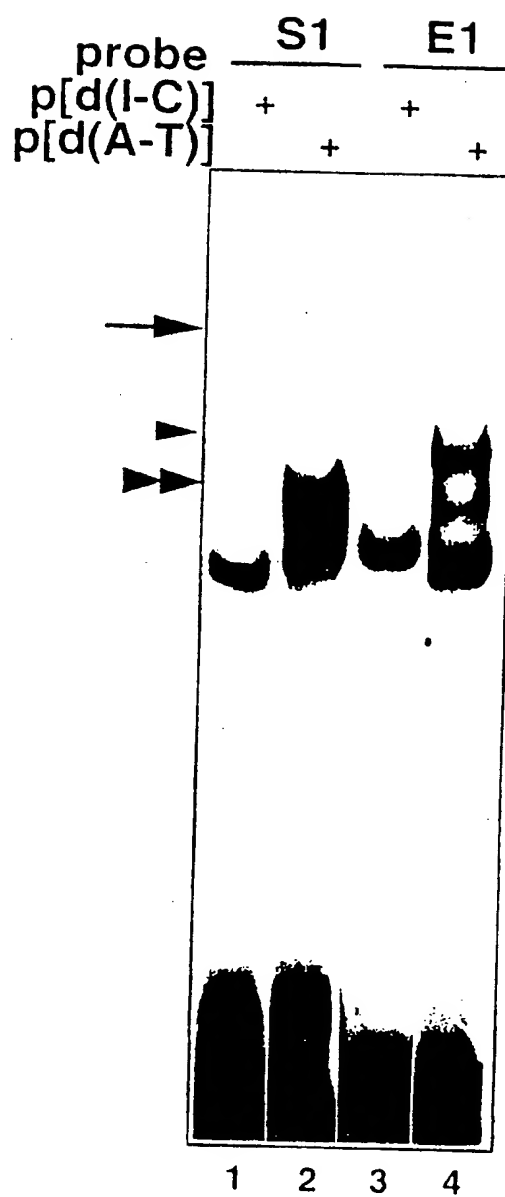


Fig. 5



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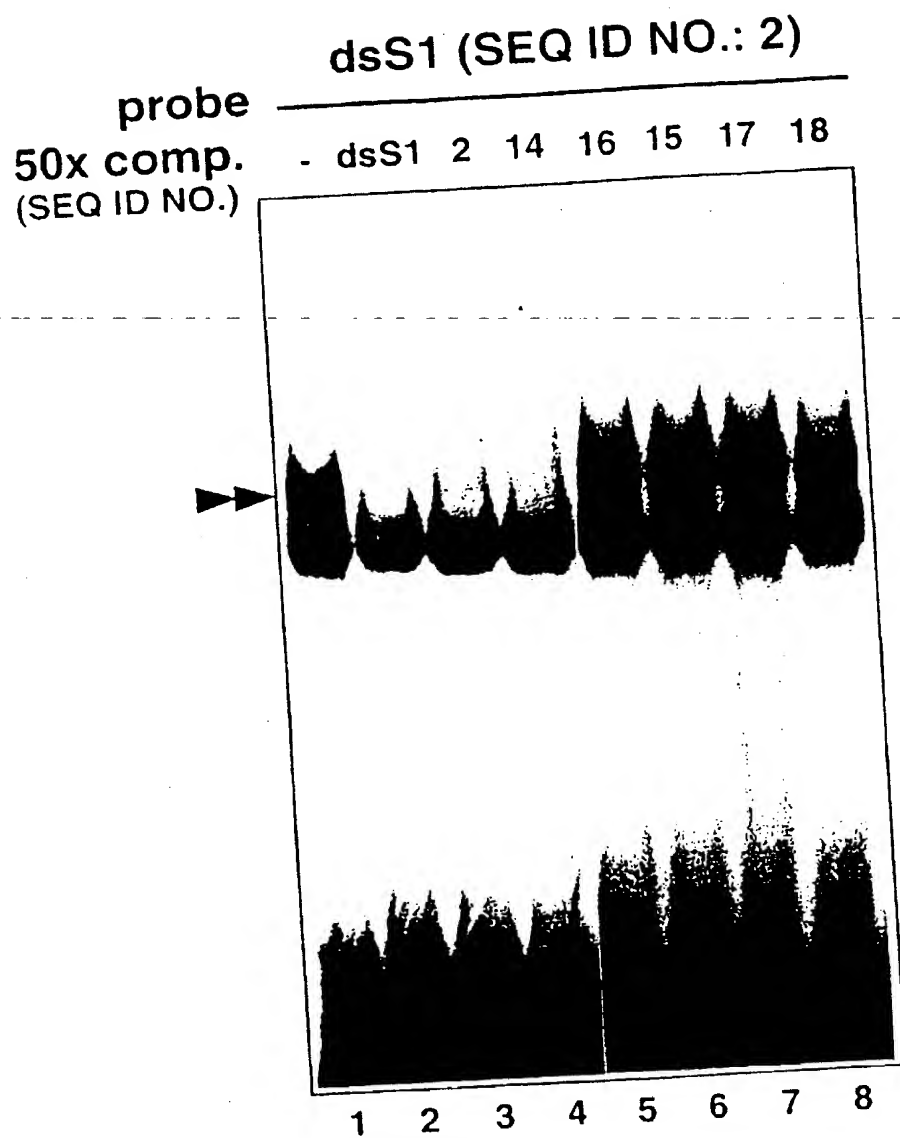


Fig. 6

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extract L929  
probe E1  
100x comp. - E1

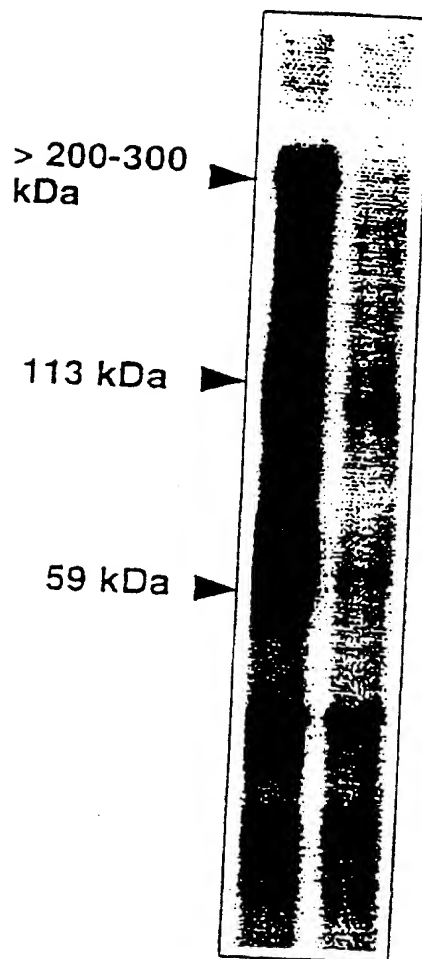


Fig. 7A

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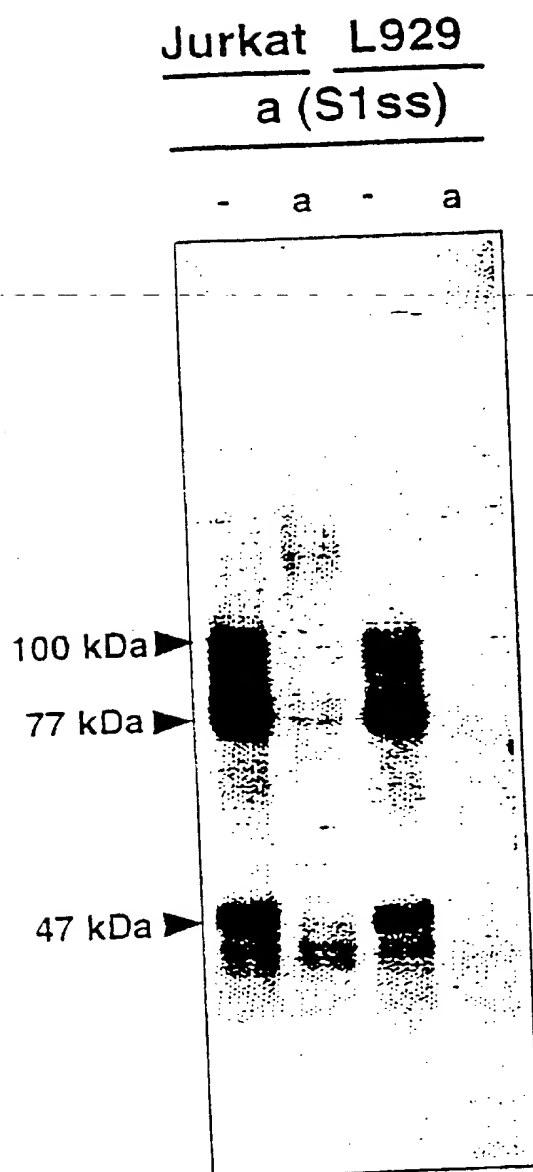


Fig. 7B

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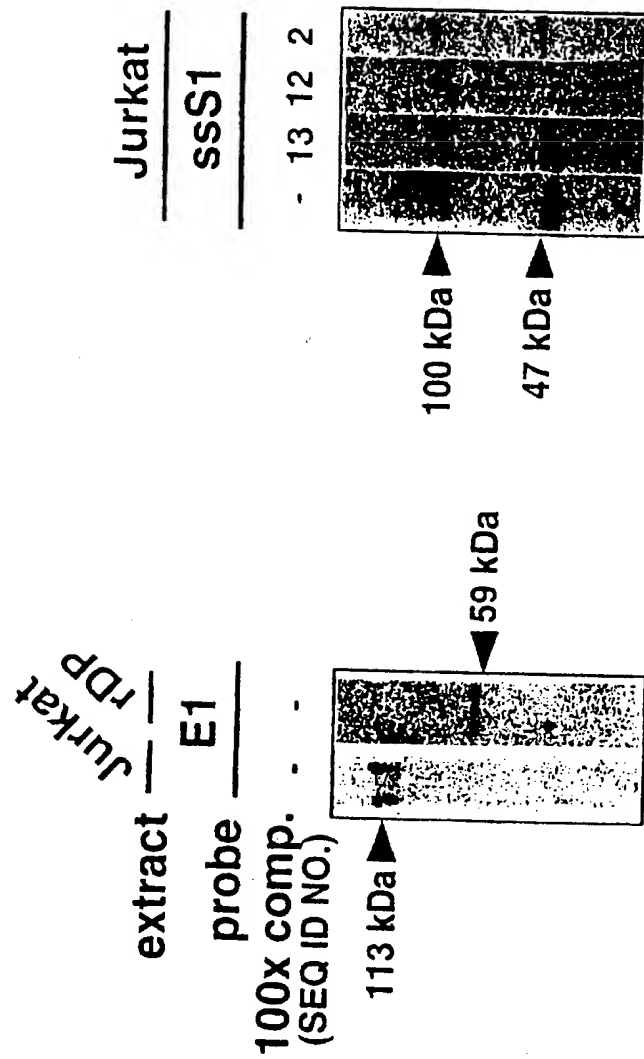
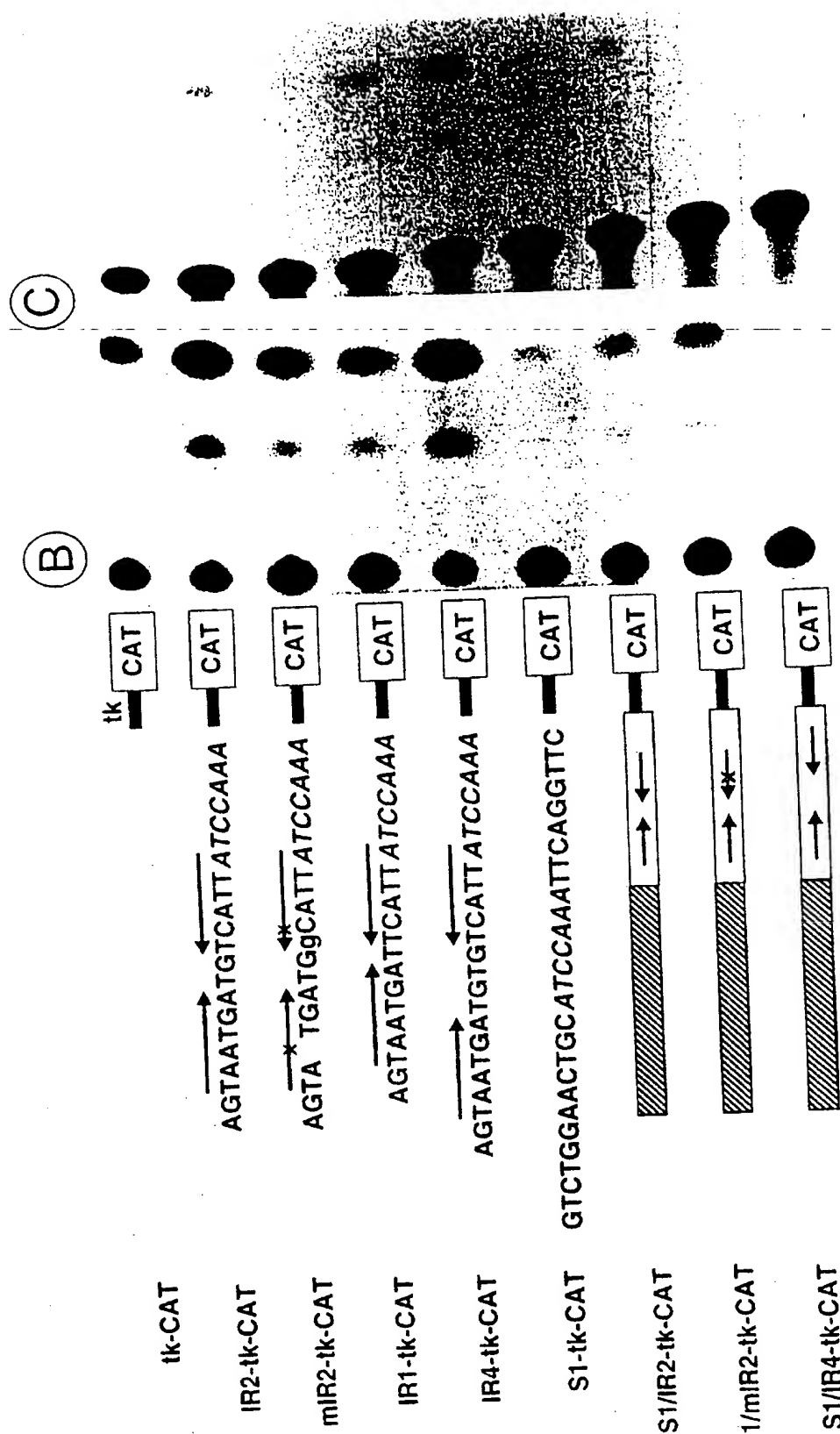
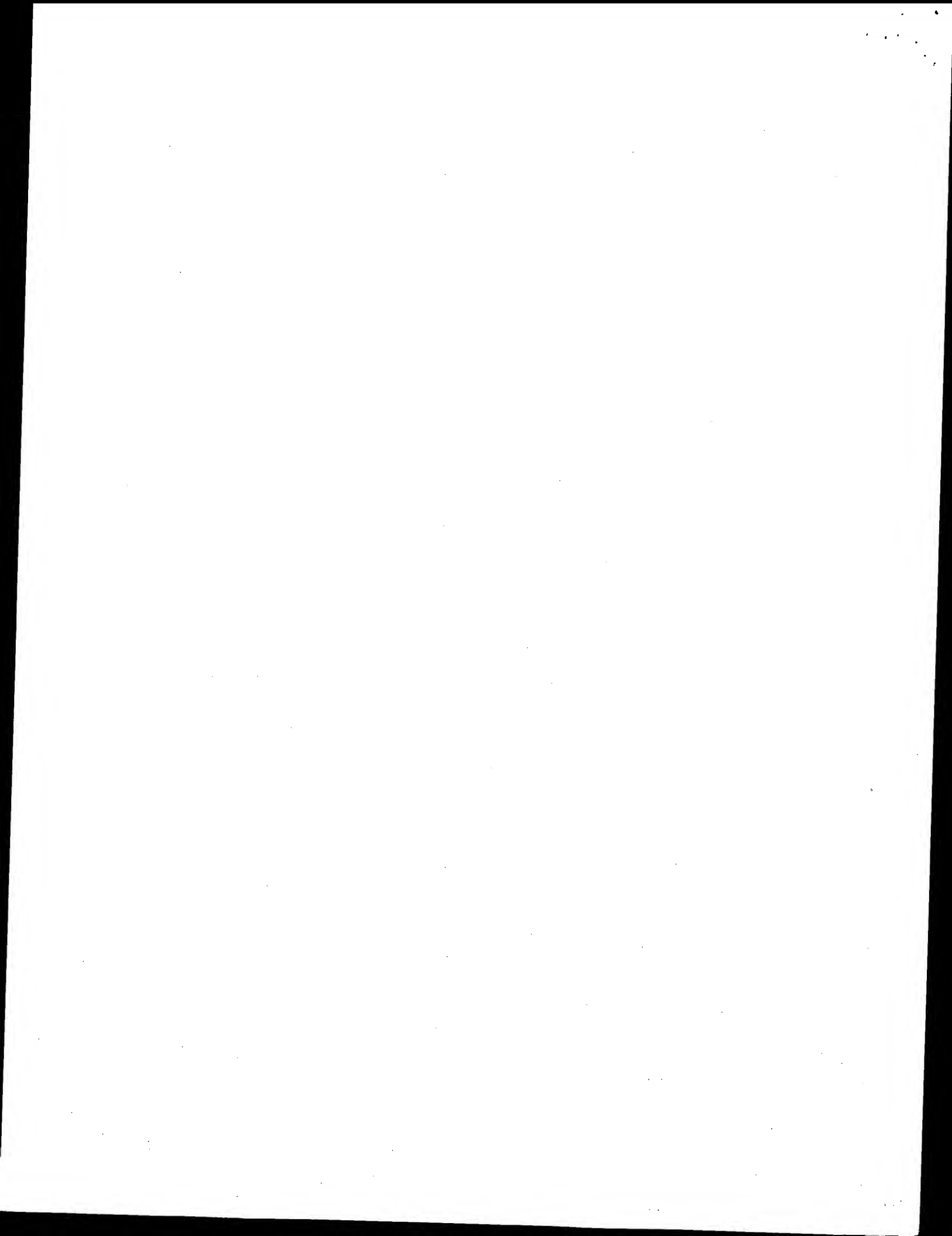


Fig. 8A

Fig. 8B





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## INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification <sup>6</sup> : <b>C12N 15/85, C07K 14/47, A61K 31/70, 38/17 // C07K 14/705</b>		<b>A3</b>	(11) International Publication Number: <b>WO 98/08965</b> (43) International Publication Date: <b>5 March 1998 (05.03.98)</b>
(21) International Application Number: <b>PCT/NZ97/00107</b> (22) International Filing Date: <b>29 August 1997 (29.08.97)</b> (30) Priority Data: <b>08/713,557</b> <b>30 August 1996 (30.08.96)</b> <b>US</b> (71) Applicant: <b>GENESIS RESEARCH &amp; DEVELOPMENT CORPORATION LIMITED [NZ/NZ]; 1 Fox Street, Parnell, Auckland (NZ).</b> (72) Inventors: <b>WATSON, James, D.; 769 Riddell Road, Auckland (NZ). RUDERT, Fritz; 1/53 Amy Street, Ellerslie, Auckland (NZ).</b> (74) Agents: <b>BENNETT, Michael, Roy et al.; Russell McVeagh West-Walker, Level 5, The Todd Building, 171-177 Lambton Quay, Wellington 6001 (NZ).</b>		(81) Designated States: <b>AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, HU, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).</b>	
		<b>Published</b> <i>With international search report.</i> (88) Date of publication of the international search report: <b>7 May 1998 (07.05.98)</b>	
(54) Title: <b>CD95 REGULATORY GENE SEQUENCES AND TRANSCRIPTION FACTORS</b>			
(57) Abstract <p>Regulatory DNA sequences that silence and enhance transcription of coding portions of the CD95 gene, which is instrumental in apoptosis, are disclosed. Proteinaceous transcription factors that bind to the silencer and enhancer regulatory sequences are also disclosed and are useful for modulating the expression of CD95 or other proteins. Methods for regulating apoptosis have therapeutic and prophylactic applications for a variety of disorders, including cancer, viral and retroviral infections, neurodegenerative disorders, immune system dysfunction, and other disorders.</p>			

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# INTERNATIONAL SEARCH REPORT

Internat. Application No  
PCT/NZ 97/00107

A. CLASSIFICATION OF SUBJECT MATTER  
IPC 6 C12N15/85 C07K14/47 A61K31/70 A61K38/17 //C07K14/705

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)  
IPC 6 C12N C07K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	RUDERT F ET AL: "IDENTIFIICATION OF A SILENCER, ENHANCER, AND BASAL PROMOTER REGION IN THE HUMAN CD95 (FAS/APO-1) GENE" DNA AND CELL BIOLOGY, vol. 14, no. 11, 11 November 1995, pages 931-937, XP000571760 cited in the application	8,11,20
Y	see the whole document	1-7,9, 12,13, 21-28
	---	-/--

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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Date of the actual completion of the international search

18 February 1998

Date of mailing of the international search report

06.03.98

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Fax: (+31-70) 340-3016

Authorized officer

Olsen, L

# INTERNATIONAL SEARCH REPORT

International Application No

PCT/NZ 97/00107

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category \* Citation of document, with indication, where appropriate, of the relevant passages

Relevant to claim No.

X	BEHRMANN I ET AL: "STRUCTURE OF THE HUMAN APO-1 GENE" EUROPEAN JOURNAL OF IMMUNOLOGY, vol. 24, no. 12, 1 November 1994, pages 3057-3062, XP000571758 cited in the application see the whole document	8,11,20
Y		1-7,9, 12,13, 21-28
A		
X	WO 96 22370 A (UAB RESEARCH FOUNDATION) 25 July 1996	8,11,20
Y	see page 34, line 23 - page 35, line 33; claims 1-24; figure 2	1-7,9, 12,13, 21-28
X	WOLFFE, A.P. ET AL.: "THE Y-BOX FACTORS: A FAMILY OF NUCLEIC ACID BINDING PROTEINS CONSERVED FROM ESCHERICHIA COLI TO MAN" THE NEW BIOLOGIST, vol. 4, no. 4, 1992, pages 290-298, XP002056090 cited in the application see the whole document	10,14, 18,19
Y		15,16, 21-28
X	MA, Z.W. ET AL.: "CONSERVATION IN HUMAN AND MOUSE PUR-ALPHA OF A MOTIF COMMON TO SEVERAL PROTEINS INVOLVED IN INITIATION OF DNA REPLICATION" GENE, vol. 149, 1994, pages 311-314, XP002056091 see the whole document	10,14, 17-20
Y		15,16, 21-28
X	BERGEMANN A D ET AL: "SEQUENCE OF CDNA COMPRISING THE HUMAN PUR GENE AND SEQUENCE-SPECIFIC SINGLE-STRANDED-DNA-BINDING PROPERTIES OF THE ENCODED PROTEIN" MOLECULAR AND CELLULAR BIOLOGY, vol. 12, no. 12, December 1992, pages 5673-5682, XP000616071 see the whole document	10,14, 18-20
Y		15,16, 21-28

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# INTERNATIONAL SEARCH REPORT

International Application No  
PCT/NZ 97/00107

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
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Y	see the whole document	15,16, 21-28
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